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Mechanisms of CRISPR-Cas9-Mediated Gene Regulation in Pseudomonas aeruginosa PAO1

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ABSTRACT

The genome editing tool, known as CRISPR-Cas9, is a powerful tool that can be employed in the fight against the multidrug-resistant opportunistic pathogen, Pseudomonas aeruginosa PAO1, which is known for causing severe infections. This review will explore the use of the CRISPR-Cas9 system in learning more about the bacterium and coming up with new ways of fighting infections. We will also look at the basic components of the CRISPR-Cas9 system, which include the Cas9 nuclease activity, specificity of the RNA, and the DNA repair machinery, which includes both homologous recombination and non-homologous end joining. We will also look at the different components of the system in the context of bacteria. This review also explores the different variants of the system, which include the catalytically dead Cas9 (dCas9) for gene silencing and the use of the system for overexpressing specific genes, known as the CRISPR activation (CRISPRa) system. This can be employed in the context of altering specific genes involved in antibiotic resistance, efflux pumps, biofilm, and virulence. We also discuss how effective it is in reversing antimicrobial resistance by targeting and degrading the genes for antimicrobial resistance, eliminating the plasmids, and making the drug-resistant strains susceptible to normal antibiotics. We also discuss the new applications for epigenetic modifications, genome-wide functional screening, and the fusion of these with phage therapy and nanoparticles. Although there have been many improvements in the field, there are still challenges in finding the most effective drug delivery methods, reducing side effects, and the ability of P. aeruginosa to alter its genes. Future perspectives include combining CRISPR-Cas9 with other cutting-edge technologies to develop synergistic approaches for combating this resilient pathogen and solving the antimicrobial resistance crisis.

INTRODUCTION

The initial role of CRISPR-Cas systems as adaptive immune systems which defend against mobile genetic elements like bacteriophages has been expanded to show their function in bacterial physiology which extends beyond their ability to detect foreign DNA [1]. Recent studies on Pseudomonas aeruginosa PAO1 have revealed its ability to use CRISPR-Cas systems for endogenous gene regulation which impacts biofilm formation and virulence manifestation [2]. CRISPR-Cas systems regulate endogenous gene expression by enabling CRISPR systems to attack self-genes which then permits pathogens to escape host defense mechanisms [3]. P. aeruginosa CRISPR-Cas systems create transcriptional interference and targeted mRNA cleavage to alter metabolic and stress response genes while promoting immune system evasion [4]. The regulatory processes of P. aeruginosa exhibit their ability to adapt to different situations while these systems enable the bacterium to maintain its pathogenic abilities and capacity to thrive in various environments [5]. The Type I-F CRISPR-Cas system of P. aeruginosa PA14 contains six "cas" genes which operate with two CRISPR arrays to produce a system that responds to biotic and abiotic stimuli through inducible expression [6]. This inducible expression profile highlights CRISPR-Cas's complex role in coordinating intricate bacterial responses to dynamic

environmental cues, establishing it as a critical factor in *P. aeruginosa* pathogenesis and adaptability. The regulation enables scientists to observe bacteria work while creating new treatment options through its mechanism of CRISPR interference which silences specific genes by targeting their DNA sequences [7]. The CRISPR interference systems which utilize catalytically inactive Cas9 (dCas9) or other Cas effectors provide P. aeruginosa researchers with dependable tools for achieving sequence-specific gene silencing through precise transcriptional repression that avoids double-strand break formation [8]. The method becomes valuable for discovering essential gene functions because it detects essential and conditionally essential genes which bacteria need to maintain antibiotic resistance and virulence during medical treatment of P. aeruginosa infections [9]. The programmable systems provide the capability to disrupt gene expression which leads to more complete understanding of how genetic factors cause this pathogen to remain harmful in its natural environment and cause diseases [10].

The widespread existence of various CRISPR-Cas systems in bacterial and archaeal domains, such as the Type I-C, Type I-E, and Type I-F systems found in *P. aeruginosa* clinical isolates, highlights their fundamental function in bacterial defense against invasive genetic elements such as phages and plasmids [3], [11]. Adaptive or heritable immunity is produced by this adaptive immune system, which is made up of a genomic CRISPR array with short sequences called spacers that were obtained from previously encountered foreign genetic materials [6]. CRISPR RNAs (crRNAs) coordinate this adaptive ability by directing Cas proteins to identify and cleave complementary foreign nucleic acids, thereby offering a strong defense mechanism [12]. Understanding the evolutionary arms race between bacteria and their genetic parasites requires more investigation into the precise mechanisms of spacer acquisition and the ensuing Cas protein-mediated interference against various mobile genetic elements in *P. aeruginosa* PAO1. According to recent research, a naturally occurring mechanism for host gene regulation is provided by nuclease-free Type IV-A CRISPR-Cas systems, which are found in a number of Pseudomonas species and also serve as CRISPR interference systems [13]. The discovery of anti-CRISPR proteins, which function as on-off switches for gene editing, further complicates and improves our understanding of CRISPR-Cas mediated regulation, highlighting potential therapeutic avenues for controlling CRISPR activity, even though these systems primarily provide sequence-specific gene silencing [14]. The complex mechanisms by which CRISPR-Cas9 systems mediate gene regulation in *Pseudomonas aeruginosa* PAO1 will be examined in this review, with a particular emphasis on how these systems can be engineered for precise genetic manipulation and their implications for comprehending bacterial pathogenesis and creating new antimicrobial strategies. Additionally, it will assess critically the possibility of using CRISPR-Cas9 for therapeutic interventions against multidrug-resistant *P. aeruginosa*, taking into account both targeted depletion of antibiotic resistance determinants and genome editing to reduce virulence [15]. The native roles of CRISPR-Cas systems in *P. aeruginosa* and the novel uses of engineered CRISPR-Cas9 tools for targeted genetic interventions will be distinguished in this investigation [16]. We will explore how this dual approach, where there is a comprehension of the inherent role of CRISPR-Cas, as well as external interventions of CRISPR-Cas9, can lead to innovative solutions to overcome *P. aeruginosa* infections, especially those that have shown resistance to conventional antibiotic treatments [17]. This includes exploring how CRISPR-Cas9 can be harnessed to specifically target efflux pumps, disable resistance genes, or even make bacterial membranes susceptible to conventional antibiotic treatments, thus overcoming common resistance mechanisms [18]. This is especially promising to revive conventional antibiotic treatments, making even multidrug-resistant bacteria susceptible to these treatments again [19,20]. This review will explore how CRISPR-Cas can help overcome antibiotic resistance by specifically targeting and eliminating resistant bacterial strains, including their resistance mechanisms, such as carbapenem-resistant plasmids [21], [22].

Overview of Pseudomonas aeruginosa PAO1 as a Pathogen

Pseudomonas aeruginosa PAO1, a paradigmatic model commonly used in experimental studies, represents a central model in elucidating the intricate pathogenesis of this opportunistic pathogen that is notoriously involved in causing healthcare-associated infections owing to its intrinsic resistance mechanisms and biofilm-forming capacity [23], [24]. This Gram-negative bacterium represents a significant clinical problem, especially in immunocompromised patients and cystic fibrosis patients, in whom the multidrug-resistant phenotype of this pathogen complicates its clinical management and contributes to high case mortality rates [25], [26]. The high metabolic adaptability of this bacterium in thriving in a wide range of environments complicates its clinical management, as it adapts easily to various environmental stressors, such as antimicrobial agents [8]. Its genomic flexibility and its complex control systems are responsible for its high adaptability and for its capacity to acquire resistance genes and to conduct complex intercellular communication [27]. Its biofilm-forming potential also contributes to its resistance to antibiotic therapy and requires novel methods to break up such biofilms [28]. Significantly, efflux pump systems and low membrane permeability are responsible for its intrinsic resistance and render most antibiotics ineffective against it [29]. The complex interrelationship between these intrinsic and acquired resistance mechanisms, such as antibiotic targets and resistance plasmids, represents a significant hurdle in antimicrobial therapy [30].

Considering this therapeutic imperative, the precise molecular basis of resistance in *P. aeruginosa* PAO1 is critical to develop targeted therapeutic approaches that can effectively overcome these intricate resistance mechanisms [31]. For example, biofilm production is one such virulence factor that compromises antibiotic efficacy and host immune response, thereby rendering infections with this bacterium difficult to manage [32]. This intricate network of resistance determinants, coupled with its

ability to evolve rapidly during chronic infections, emphasizes the need to develop novel therapeutic approaches against *P. aeruginosa* infections [33, 34]. Therefore, using sophisticated biotechnological tools such as CRISPR-Cas9 to specifically modulate these resistance determinants is critical to overcome the formidable therapeutic challenges posed by *P. aeruginosa* infections [35]. Indeed, the complex molecular processes underlying multidrug resistance in *P. aeruginosa*, such as efflux pumps, enzymatic inactivation, and biofilm production, require the investigation of particular genomic alterations [36, 37]. To illustrate, the low permeability of its outer membrane, efflux pumps, production of antibiotic-inactivating enzymes, and production of metabolically inert persister cells within biofilms all contribute to its remarkable resistance profile [38, 39, 40]. These processes, together with horizontally derived resistance determinants and adaptive responses to environmental stress agents, enable *P. aeruginosa* to evade many different antimicrobial agents [41, 42, 43]. These multifaceted resistance processes, such as deficiencies in OprD membrane proteins and overproduction of beta-lactamase enzymes, greatly undermine the effectiveness of current antimicrobial agents [44]. Consequently, innovative therapeutic modalities that specifically target such resistance processes are critically needed for the resensitization of bacteria and the improvement of patient outcomes. Specifically, carbapenem resistance in *P. aeruginosa*, an area of critical priority as defined by the World Health Organization, has been associated with extensive co-resistance to a wide array of other antibiotics. This critical resistance phenotype is often associated with chromosomal mutations affecting permeability and/or the acquisition of metallo-beta-lactamase genes and thus requires the application of targeted genetic interventions [46], [47].

CRISPR-Cas Systems: Beyond Adaptive Immunity

Initially, these CRISPR-Cas systems were classified as prokaryotic adaptive immune systems, which have exceeded their natural function, showing remarkable potential as precise gene editing tools to disable particular bacterial genes, thus reducing multidrug resistance [48, 49]. This is due to their inherent ability to specifically target DNA or RNA, thus showing remarkable precision in manipulating bacterial genomes to combat antibiotic resistance, such as efflux pumps and biofilm production [50, 51]. This includes their ability to engineer novel antimicrobial agents that specifically degrade resistance-conferring plasmids or chromosomes, thus showing a paradigm shift in combating resistant infections [52]. The ability of CRISPR-Cas systems to adapt to reprogram antibiotic resistance further shows their potential, especially in situations where conventional antibiotics have failed [53].

The Promise of CRISPR-Cas9 in Bacterial Gene Regulation

The programmable ability of CRISPR-Cas9, facilitated by a customizable single guide RNA (sgRNA), allows precise targeting of particular genetic loci of bacterial pathogens such as *P. aeruginosa*, providing a direct method to counteract virulence factors or resistance determinants [20], [24]. This precise method is highly promising to overcome antibiotic resistance issues, providing innovative methods to combat drug-resistant tuberculosis infections [54]. CRISPR/Cas9 is designed to target genes that confer antibiotic resistance or virulence factors, leading to cell death or increased susceptibility to existing antimicrobial agents [55]. Moreover, precise CRISPR-Cas9 modifications can modulate efflux pump genes, such as those of the MexAB-OprM system, to reestablish antibiotic levels within bacterial cells to therapeutic levels [56]. This precise genetic manipulation is not limited to disrupting biofilm-forming genes, which is a major factor in chronic P. aeruginosa infections, thus making these bacterial populations more susceptible to eradication [57]. Apart from gene disruption, the versatility of CRISPR-Cas systems is not limited to gene regulation using CRISPR interference and CRISPR activation, which can precisely up- or downregulate gene expression without any permanent changes to the genome [21]. This precise regulation of gene expression provides a sophisticated tool to fine-tune bacterial physiology, which could potentially increase antibiotic efficacy by making the pathogens more sensitive or altering their metabolic states. Additionally, CRISPR-Cas tools can be harnessed to selectively eliminate plasmids that harbor antibiotic resistance genes, thus reversing the process of resistance phenotypes in problem-causing bacterial strains [58]. These targeted approaches, exemplified by the CRISPR-Cas9 tool, provide a novel method to tackle drug resistance in respiratory disease, including that caused by *P. aeruginosa* [54], [59].

Fundamentals of CRISPR-Cas9 Systems

The main components of the system include Cas9 endonuclease, which acts as a nuclease enzyme, and a customizable guide RNA that targets a specific gene in the bacterial genome [60]. The Cas9 enzyme then cleaves the targeted DNA strand in a process that leads to gene disruption or homologous recombination for gene editing [54]. This system allows for a direct attack on genes that encode resistance and virulence factors [24]. The specificity of this system represents a novel route for managing antibiotic resistance gene content and eliminating pathogens in a unique and specific manner [61]. In addition, the potential for engineering Cas9 to target multiple genomic sites in a cell by delivering multiple guide RNA molecules represents a promising route for this system's application in therapy due to its potential to disrupt multiple resistance mechanisms simultaneously [43].

Components of CRISPR-Cas9: Cas9, sgRNA, and Protospacer Adjacent Motif (PAM)

Cas9 endonucleases have a characteristic RuvC domain and HNH nuclease, which enable cleavage of both strands of DNA upon accurate hybridization of the guide RNA with the protospacer sequence, flanked by a Protospacer Adjacent Motif. This recognition of the PAM sequence is essential for Cas9 activity, as its absence prevents cleavage, thus protecting the host genome from self-targeting [62]. The single guide RNA (sgRNA) is a synthetic RNA that comprises a fusion of CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA) and is essential for specificity, with its 20-base spacer sequence

complementary to the desired genomic sequence. Such intricate molecular specificity allows for the very precise targeting of bacterial resistance genes/virulence factors, which can be subsequently disrupted/modified for therapeutic use. The series of events, from the import of the protospacer sequence into the CRISPR array, followed by its transcription into a precursor transcript RNA, and subsequent processing into mature crRNA with the aid of RNase III, ultimately leads to the creation of the active Cas9 ribonucleoprotein complex [61].

This complex then critically engages with the target DNA through PAM-dependent recruitment, which leads to the initiation of R-loop formation, where sequence complementarity is facilitated by the sgRNA, thus allosterically activating the HNH domain of the nuclease to cleave the dsDNA [63]. This intricate recognition process, dependent on the sgRNA-DNA complex, is critical for specificity, which is essential in differentiating between various bacterial strains, especially during therapeutic interventions [64]. This specificity is essential, especially in the design of CRISPR-based antimicrobial agents that can effectively eliminate various pathogenic bacteria, including those that harbor resistance genes, without affecting commensal bacteria [65]. Designing chimeric guide RNAs (gRNAs) is critical, especially in choosing inducible expression systems, which is essential in optimizing CRISCR-Cas9 efficacy, especially in bacterial systems, especially for antimicrobial use [66].

The architectural flexibility of guide RNA, especially through the chimeric fusion of crRNA and tracrRNA to form a single guide RNA (sgRNA), further rationalizes the Cas9 assembly process, which transforms an inactive Cas9 into an active form that is capable of sequence-specific targeting [67], [68]. This precise targeting system, therefore, allows for discriminative disruption of detrimental genetic elements while maintaining the integrity of the host's genetic information [69]. This is an innovative strategy that holds immense promise for the design of specific and effective therapeutic agents for combating MDR strains of *P. aeruginosa*. The efficiency and specificity of the Cas9-catalyzed cleavage event are significantly affected by the specific sequence of the protospacer adjacent motif and its interaction with the Cas9 enzyme. This varies for different Cas9 orthologs [70]. For example, the most widely used Streptococcus pyogenes Cas9 recognizes the NGG PAM sequence, which is essential for the initiation of DNA binding and subsequent double-stranded cleavage [71].

Mechanisms of DNA Cleavage and Repair Pathways

After the formation of the active ribonucleoprotein complex and the cleavage of the DNA, the cellular response is primarily carried out by two distinct DNA repair mechanisms: non-homologous end-joining and homology-directed repair. In bacteria, the repair of DNA by the homology-directed mechanism, with the use of exogenous templates and λ-red recombination, can result in precise gene insertions or corrections, while non-homologous end-joining results in insertions or deletions, leading to the disruption of gene function. This level of precision in gene editing, particularly by the homology-directed repair mechanism, can be a potent approach in the development of new antimicrobial agents [74]. The use of CRISPR-Cas9 in P. aeruginosa utilizes these repair processes, either to knockout genes or to introduce desired genetic changes, thus providing a sophisticated approach to deal with its notorious ability to adapt and develop resistance [75]. More specifically, the Cas9 protein, which is an RNA-guided DNA endonuclease, locates precise sites within the genome according to RNA complementarity, including a protospacer adjacent motif, resulting in double-strand DNA breaks that are then repaired by the cell's inherent repair processes [76], [77]. Even more specifically, the specificity of Cas9 binding is determined by the requirement for a protospacer-adjacent motif, which is a sequence of DNA adjacent to the site recognized by the Cas9 enzyme [78]. This critical dependence on PAM ensures that Cas9 only cleaves foreign DNA, thus protecting the bacterial host genome from self-inflicted damage [79]. The PAM sequence recognized by Cas9 can vary considerably between different Cas9 orthologs, affecting the number of genomic loci available for editing, as well as the risk of off-target effects [80, 81]. The DNA double-strand breaks created by Cas9 cleavage of target DNA are mainly repaired by homology-directed repair or nonhomologous end-joining in eukaryotic cells. However, in most bacteria, including P. aeruginosa, nonhomologous end-joining is absent, leaving homology-directed repair as the main pathway for repairing DNA double-strand breaks [83]. This dependence upon homology-directed repair in P. aeruginosa provides a unique advantage in genome editing by favoring the use of exogenous DNA templates for gene editing compared to error-prone repair mechanisms [84]. Thus, optimization of repair template delivery and control of homologous recombination enzyme activity are important for maximizing genome editing in *P. aeruginosa* [85]. The absence of non-homologous end-joining in most prokaryotes, including *P. aeruginosa*, makes DNA strand breaks highly lethal in these organisms, thereby underlining the importance of homology-directed repair in genome editing by the use of CRISPR-Cas systems [62]. This divergence in repair mechanisms between prokaryotes and eukaryotes requires different approaches to genome editing by the use of the CRISPR-Cas9 system for gene knockout and gene editing in prokaryotes and eukaryotes, respectively [86], [87]. In eukaryotes, however, this repair mechanism is more efficient and prevalent but often results in insertions and deletions that affect gene functions and require consideration in genome editing strategies [88], [89].

Engineered CRISPR-Cas9 for Gene Editing and Regulation

Protein engineering of Cas9 has emphasized creating Cas9 variants with modified PAM recognition sites, extended target site ranges, and improved cleavage activity to address the challenges of using native Cas9 systems [90]. These modifications have included creating high-fidelity Cas9 variants that minimize off-target effects, while smaller Cas proteins have enabled efficient delivery of these proteins into bacterial cells [91]. Additionally, catalytically dead Cas9 (dCas9) that is fused with transcriptional activators or repressors enables regulation of gene expression without creating double-strand breaks,

thus providing a reversible system to regulate gene expression in P. aeruginosa. This sophisticated regulation of gene expression using CRISPR interference and CRISPR activation is a tool to explore gene function and virulence factors in P. aeruginosa, even without altering its genome. These sophisticated tools of CRISPR-Cas9 technology, therefore, hold promise to help decipher the intricate regulatory machinery in P. aeruginosa and to develop new anti-pseudomonal therapies. Development of tools to increase the efficiency of homologous recombination while simultaneously downregulating non-homologous end-joining pathways can further fine-tune precise genome editing in P. aeruginosa, allowing for more refined genetic manipulations [93], [94]. For example, exploiting heterologous non-homologous end-joining pathways has been shown to increase transformation efficiency and reduce deletion size in certain bacterial pathogens, which can potentially be extended to P. aeruginosa to fine-tune genome editing [95]. Furthermore, engineering expression of particular DNA repair enzymes or synthetic pathways can also help to fine-tune precision in genome editing mediated by the CRISPR-Cas9 system in this pathogen [96].

Comparison with Other Gene Editing Technologies

Although CRISPR-Cas9 has greatly transformed genome editing through its unparalleled efficiency and cost-effectiveness, it is imperative to contextualize these benefits and drawbacks against other genome editing tools such as zinc finger nucleases and transcription activator-like effector nucleases [97]. These tools, despite their foundational importance, have intricate design parameters, which have greatly limited their use, especially in complex bacterial genomes such as P. aeruginosa [98]. Conversely, the RNA-guided targeting mechanism of CRISPR-Cas9 is highly flexible, allowing facile reprogramming of specificity, thus greatly accelerating the rate of genetic investigation and manipulation of this opportunistic pathogen [7]. This versatility has led to the design of high-throughput genetic screens, which were previously not possible using other genome editing tools.

CRISPR-Cas9 for Transcriptional Repression: CRISPRi

Mechanism of dCas9-Mediated Transcriptional Interference

The use of a catalytically dead Cas9 protein fused with repressor domains or dCas9 sterically hindering RNA polymerase for sequence-specific transcriptional gene silencing does not alter the genomic DNA sequence. This is mediated by the specific targeting of dCas9 to specific genes or promoter regions, thus impeding the process of transcription initiation or elongation. This is mediated by mutations in key active sites in the Cas9 protein, namely Asp10 and His840, which generate a dCas9 enzyme that is catalytically inactive but retains its specificity in DNA binding. This dCas9 protein, mediated by a single guide RNA (sgRNA), binds to the target DNA and physically blocks the process of transcription or gene expression. This is an advantage in the reversible control of gene expression, making it an essential feature in the analysis of essential genes or those genes in complex networks where the knockout of genes is lethal or produces ambiguous phenotypes. CRISPRi systems have been successfully implemented in P. aeruginosa to downregulate essential genes such as prtR that cannot be destroyed by gene knockout methods and have provided insights into understanding the functions of such genes in bacterial virulence and metabolism [8]. Thus, this non-destructive gene silencing capability makes it a valuable tool in understanding the functions of important genes in bacterial pathogenesis and antibiotic resistance, especially in cases where gene knockout is lethal [9]. In addition, this tool allows for gene dosage effects and transcriptional dynamics, providing insights into the subtle mechanisms that govern bacterial biology and pathogenesis [103].

Targeting Promoters and Coding Regions for Gene Silencing

CRISPR interference systems have been shown to attain gene repression by directing dCas9 to either the promoter region, which prevents RNA polymerase binding, or to the coding region, which prevents transcriptional elongation [104]. In addition, repression is dependent upon the precise location of the sgRNA, such that maximum repression is attained if the sgRNA is placed downstream of the transcription start site or within the initial codons of the gene [105], [106]. Targeting the non-template strand of a gene or both strands of its promoter region has been shown to attain maximum knockdown efficiency in bacterial cells [107]. Furthermore, the use of various sgRNAs that target different regions within a gene may provide a more refined depth and degree of gene transcription repression [108]. For instance, it has been shown that the physical interaction between the dCas9-sgRNA-DNA and RNA polymerase-DNA complexes is a major mechanism in transcriptional repression [109]. In addition to this mechanism, dCas9-sgRNA-DNA binding in promoter regions may interfere with the recruitment of transcription activators and RNA polymerase itself, thereby providing a second mechanism for gene transcription repression [110]. Such a refined mechanism in gene expression allows for high-throughput functional genomics screening to identify important genes involved in various physiological functions, such as antimicrobial resistance and virulence [111].

Applications of CRISPRi in P. aeruginosa PAO1

CRISPRi has thus been established as a potent method for conducting functional genomic studies in P. aeruginosa PAO1, facilitating the study of gene essentiality and phenotypic response to gene knockdown [112, 113]. Through this method, researchers have the ability to regulate levels of gene expression, thus revealing intricate details about gene regulation, especially those essential for survival under certain environmental pressures or infection of a host [114]. This is further enhanced by the creation of sgRNA libraries, which facilitate genome-wide screens, thus revealing novel targets for therapy, as well as intricate details about gene networks. For example, CRISPRi screens have played a critical role in revealing genes associated with antibiotic resistance, thus providing critical insights into P. aeruginosa infection. Furthermore, this method provides precise regulation, thus facilitating the study of gene function in a dosage-dependent manner, thus providing a more precise understanding compared to

knockout studies. This allows for the comprehensive mapping of the bacterial fitness landscape in response to different selective pressures, including antibiotics, based on the relationship between the knockdown of particular genes and the observed changes in the bacterial phenotype [117]. This strategy enables the identification of new, uncharacterized genes involved in antibiotic tolerance, which could be useful as targets for new antimicrobial agents [118].

Challenges and Optimization of CRISPRi for Bacterial Systems

However, although CRISPRi has significant benefits, it faces challenges in various bacterial systems, including P. aeruginosa, in terms of efficient delivery systems for dCas9 and sgRNA, off-target effects, and optimization of guide RNA design [119], [120]. Overcoming these challenges may require the development of novel delivery systems such as phagemids and/or integrative plasmids and/or developing powerful computational tools that enable the design of extremely specific guide RNAs to minimize off-target effects and potential regulatory effects. In addition, the inherent genetic flexibility and various metabolic capabilities of P. aeruginosa [8] require consideration of various bacterial-specific factors that may affect the efficiency of the CRISPRi system in each bacterial background, such as endogenous nucleases and/or different transcriptional profiles between bacterial strains. Such challenges require optimization of each bacterial system by selecting appropriate variants of dCas9 and inducible promoters that minimize off-target effects and/or pleiotropy and/or maximize specificity. To this end, various approaches, such as codon optimization of dCas9, together with the use of synthetic sgRNA, have been employed to improve the levels of expression of CRISPRi system components in P. aeruginosa. These developments not only ensure the use of CRISPRi in basic research endeavors, but also provide tools for the design of innovative approaches to combat antibiotic resistance, especially in resensitizing MDR strains to conventional antibiotics [121], [122], [123]. Furthermore, efforts to develop novel dCas9 variants with improved DNA specificity, such as expanded PAM SpCas9, have shown promise in minimizing off-target effects, a major problem that is inherent in genome-wide CRISPRi studies, especially in bacteria [80]. These developments in CRISPRi system tools have greatly improved our ability to address issues related to antibiotic resistance, especially in recalcitrant Gram-negative bacteria such as P. aeruginosa [15], [124]. Other innovative tools have been developed, especially with regard to gene regulation, such as translation regulation, where CRISPR-Cas13, which uses RNA-binding proteins, is employed to regulate mRNA translation levels [125]. Most gRNA design tools have focused on CRISPR-Cas9, although the general principle is often applicable to other Cas proteins, such as Cas12 and Cas13.

CRISPR-Cas9 for Transcriptional Activation: CRISPRa

Unlike CRISPRi, where a catalytically dead Cas9 (dCas9) is fused with transcription activators, CRISPR activation allows for the upregulation of gene expression, providing a distinct strategy for studying gene functions and engineering metabolic pathways [104]. This technology, referred to as CRISPRa, allows for the overexpression of target genes, providing a tool for studying gene dosage effects and for building complex gene expression circuits for synthetic biology applications [127, 128]. The CRISPRa approach utilizes a catalytically dead Cas9 (dCas9) fused with effector proteins that recruit transcription machinery, initiating gene transcription. For bacterial cells, the CRISPRa approach is based on a catalytically dead Cas9 (dCas9) fused with a variety of transcription activator domains, such as the ω subunit of RNA polymerase or response regulators of two-component bacterial signal transduction systems, for the recruitment of RNA polymerase to target promoters.

Mechanisms of dCas9-Mediated Transcriptional Activation

This targeted recruitment is advantageous in facilitating the initiation of transcription and presents a precise method for modulating gene expression levels and studying gene functions by overexpressing them [131]. The versatility of this application of CRISCRa is extended to multiplexed activation, which allows for simultaneous activation of multiple genes and is advantageous in optimizing metabolic pathways and studying gene interactions in complex gene networks [132]. However, the efficacy and wide application of bacterial CRISCRa have been limited by the limitations in activation potential and versatility and have therefore been a subject of ongoing research into novel dCas9 fusion proteins and guide RNA to overcome these limitations [101], [128]. The recent developments in CRISCRa techniques have involved fusing dCas9 with various transcription activators such as the ω subunit of RNA polymerase to optimize gene expression in bacterial cells [133], [134]. For example, various architectural configurations of activator domains, such as single or multiple fusions, have been tested to optimize transcriptional output in various bacterial backgrounds [135, 136]. This includes exploiting well-characterized transcriptional activators such as VP64, p65AD, or Rta, alone or in combination, fused to dCas9, to effectively recruit the transcriptional machinery to the targeted gene promoter regions [137, 138]. In prokaryotic organisms, this is achieved by fusing dCas9 with bacterial activator domains, such as the SoxS protein, which has shown significant levels of mRNA production when correctly positioned near the promoter regions of genes [139]. Importantly, the precise positioning of guide RNAs targeting particular promoter regions is critical to optimize CRISRa activity, with studies showing that targeting within a 200 bp upstream to 1 bp downstream region of the transcription start site is required to achieve maximum activity [140]. This can be further refined to a 10- to 11-base peak activity, which reflects a single DNA helix turn relative to the transcription start site [126]. In addition to dCas9-based systems, other catalytically dead Cas proteins, such as dCas12a and dCas12f, are being considered as potential tools in CRISPRa in bacteria, which can provide new PAM specificities as well as potentially more compact designs to achieve efficient gene activation [141, 142]. Alternative Cas proteins, such as dCas12a, which can potentially drive gene activation, have shown

promise in mammalian systems, although their use in bacteria remains limited [142].

Recruiting Transcriptional Activators to Target Promoters

The already enhanced efficacy of CRISPRa systems is further increased by optimization of the recruitment of multiple transcriptional activators by utilizing a modular scaffold design or multimerization strategies such as the SunTag and MoonTag systems [143]. Such systems have been shown to be capable of recruiting multiple activator complexes to a single dCas9 molecule, thereby resulting in a synergistic effect in gene expression due to increased localized transcriptional machinery at the target gene promoter [144]. In addition, the strategic integration of MS2 aptamers into the scaffold of the sgRNA and subsequent recruitment by the MS2 coat protein have been shown to be a high-efficiency system for gene activation in vivo, especially in compact dCas12f systems [145].

Current Limitations and Future Directions for CRISPRa in P. aeruginosa PAO1

Nevertheless, the utilization of CRISPRa in P. aeruginosa PAO1 is still impeded by challenges, especially in relation to the innate regulatory intricacies in the genome. The compact and malleable design of dCas12a, in addition to its alternative PAM recognition properties, is likely to greatly increase the number of accessible genomic loci for CRISPRa in P. aeruginosa PAO1 [146]. Additionally, the selection and optimization of promoter sites as well as guide RNA are critical determinants in the utilization of CRISPRa in P. aeruginosa PAO1, as the distance from the transcription start site is critical for efficient activation [126], [132]. Further innovations in bacterial CRISPRa technology may be realized through the utilization of transcription factors as protein regulators in the form of dynamic DNA translators that change shape in response to the binding of specific transcription factors [147]. This strategy may provide more subtle and adaptive control in gene regulation, extending beyond simple ON/OFF control to modulate bacterial phenotype in a more refined manner. Such advanced control in gene regulation may provide a more refined control in bacterial metabolism, virulence factor synthesis, and biofilm formation in P. aeruginosa PAO1, providing potential avenues to combat antibiotic resistance and optimize bioproduction in this bacterium. Moreover, strategic use of CRISPR interference by utilizing a catalytically dead Cas9 enzyme or other Cas9 variants may provide a powerful strategy in precisely controlling gene expression by complementing CRISPRa and allowing systematic analysis of gene functions by controlled repression [148].

Engineering Novel Activator Domains for Bacterial Use

In order to overcome some of the limitations associated with currently existing activator domains, researchers have been working to design synthetic transcription factors and aptamer-tethered effectors that are specifically designed for bacterial intracellular contexts and have shown promise in providing better orthogonality and control in activation kinetics. This has involved designing novel RNA-guided transcriptional activators that utilize bacterial-specific mechanisms for gene expression modulation and have moved beyond utilizing protein-DNA interactions to include RNA-mediated regulation mechanisms. Moreover, utilizing epigenetic modification domains such as KRAB-DNMT3A and VPR-TET1 based on dCas9 or dCas12f backbones has shown promise in providing more sustainable gene regulation by simultaneously modulating both transcriptional and epigenetic contexts [149]. Further optimization of CRISPRa systems in P. aeruginosa PAO1 may include directed evolution of Cas proteins to reduce off-target effects and increase specificity, as well as developing novel delivery systems that are specifically designed for this unique organism's characteristics [104].

CRISPR-Cas9 for Epigenetic Modifications and Chromatin Remodeling

In addition to directly editing the genome, the CRISPR-Cas9 system can also be adapted for the induction of reversible epigenetic modifications, which can influence gene expression without altering the genome sequence [150]. This is achieved by fusing the nuclease-deactivated Cas9 (dCas9) with epigenetic enzymes, such as histone acetyltransferase or deacetylase, DNA methyltransferase, or demethylase, and directing it to specific chromosomal regions. This approach allows for the exploration of epigenetic control in bacterial cells, which may reveal new epigenetic control mechanisms for virulence and antibiotic resistance in the bacterium P. aeruginosa PAO1 [3]. This new approach for applying the CRISPR-Cas9 system, termed microbial synthetic epigenetics, is a complex system for designing synthetic biology tools that exploit the power of reversible gene control mechanisms without altering the genome sequence [151]. This approach allows for the exploration of chromatin structure and the impact of chromatin structure on gene expression in bacterial pathogens, providing a better understanding of the adaptive responses of pathogens and their virulence potential [152].

dCas9-Fusion Proteins for DNA Methylation and Demethylation

The first principle is to use a catalytically inactive Cas protein, such as dCas9, fused to an epigenetic regulator to a target locus of interest through a guide RNA, which allows for the regulation of epigenetic marks at a particular locus without causing double-stranded breaks [153]. For example, dCas9 can be engineered to carry DNA methyltransferases such as DNMT3A or ten-eleven translocation enzymes to achieve locus-specific hypermethylation or demethylation, respectively, to control gene expression through DNA methylation editing [154, 155].

CRISPR-Mediated Histone Modifications in Eukaryotic Systems: Lessons for Bacteria

The first principle is to use a catalytically inactive Cas protein, such as dCas9, fused to an epigenetic regulator to a target locus of interest through a guide RNA, which allows for the regulation of epigenetic marks at a particular locus without causing double-stranded breaks [153]. For example, dCas9 can be engineered to carry DNA methyltransferases such as DNMT3A or ten-eleven

translocation enzymes to achieve locus-specific hypermethylation or demethylation, respectively, to control gene expression through DNA methylation editing [154, 155].

Exploring Epigenetic-like Regulation in P. aeruginosa PAO1 with CRISPR-Cas9

Research in this area may be directed towards understanding the potential use of dCas9 systems in investigating the role and importance of bacterial DNA methyltransferase modification sites, especially those that are not in open reading frames and are still not well understood [158]. This may provide a better understanding of how these regions regulate gene expression by means of methylation patterns and may provide a broader understanding of gene expression control in bacterial systems.

CRISPR-Cas9 for Genome-Wide Screening and Functional Genomics

CRISPR-Cas9 technology offers a robust platform for high-throughput interrogation of gene function, enabling systematic identification of essential genes, virulence factors, and antibiotic resistance determinants in P. aeruginosa PAO1 through targeted gene knockouts or perturbations. This includes the application of CRISPR interference and CRISPR activation screens to systematically identify genes involved in specific phenotypes, such as biofilm formation or host colonization, by modulating gene expression levels rather than complete ablation. Furthermore, pooled library screens utilizing dCas9-sgRNA complexes can simultaneously assess the phenotypic impact of thousands of gene perturbations, providing a comprehensive functional map of the P. aeruginosa PAO1 genome [159]. These approaches, leveraging the versatility of various Cas systems like Cas12 and Cas13, facilitate unprecedented precision in editing, regulating, or silencing genes, which is particularly valuable in addressing complex challenges such as antimicrobial resistance [20]. For instance, comparative analysis of nuclease activity among Cas variants can identify optimal effectors for specific gene regulation tasks within P. aeruginosa, potentially unveiling novel functionalities like in vivo RNase activity [23]. Moreover, the ability to rapidly generate comprehensive genomic libraries for CRISPR-based screens allows for the identification of novel drug targets and the elucidation of complex genetic interactions that underpin bacterial pathogenesis and adaptation [24], [160]. The integration of transcriptomics and metabolomics with these genomic insights can further refine target identification by revealing system-level dynamics in response to various stressors, such as polymyxin exposure [161]. This integrated approach can provide a holistic understanding of resistance mechanisms, paving the way for synergistic interventions that re-sensitize antibiotic-resistant populations and enhance therapeutic outcomes [43].

Application of CRISPR-Cas9 in P. aeruginosa PAO1 Research

CRISPR-Cas9 serves as an invaluable tool for precise genetic manipulation and functional genomics in P. aeruginosa PAO1, enabling the generation of specific mutations, deletions, and insertions crucial for understanding gene function and regulatory networks [74].

This technology facilitates detailed investigations into pathogenicity, antibiotic resistance mechanisms, and biofilm formation by allowing for targeted genetic modifications and subsequent phenotypic characterization [32]. Specifically, CRISPR-Cas9 can be employed to dissect the roles of virulence genes, such as those involved in toxin production or immune evasion, whose disruption can attenuate bacterial pathogenicity [162]. Beyond virulence factors, CRISPR-Cas9 can also be utilized to engineer P. aeruginosa PAO1 strains with enhanced susceptibility to existing antibiotics, thereby offering a strategic approach to overcome antimicrobial resistance [22]. The development of such engineered strains could provide invaluable insights into the genetic determinants of antibiotic resistance, paving the way for novel therapeutic strategies. Furthermore, the precise manipulation capabilities of CRISPR-Cas9 extend to the development of phage resistance in P. aeruginosa PAO1, where targeted modifications can enhance bacterial evasion of phage predation [163]. This approach could also inform the design of novel antimicrobial strategies that exploit phage-bacteria interactions, potentially leading to engineered phages with enhanced lytic capabilities against resistant P. aeruginosa strains. Additionally, CRISPR-Cas9-mediated engineering can facilitate the study of efflux pump mechanisms, outer membrane permeability, and other intrinsic resistance pathways, providing a comprehensive understanding of multidrug resistance in P. aeruginosa [56], [164].

Indeed, this includes inducing specific reverse mutations in epidemic multidrug-resistant genotypes like PA154197 to restore antibiotic sensitivity [51]. This targeted re-sensitization approach could involve the precise modification of genes encoding key resistance determinants, offering a promising avenue for reversing phenotypic resistance in clinical isolates [75].

Challenges and Future Perspectives

Despite the considerable advancements, challenges remain in optimizing delivery systems for CRISPR-Cas9 components into P. aeruginosa PAO1, particularly in achieving high transformation efficiencies across diverse clinical and environmental strains [165].

Addressing this necessitates the development of novel vector systems, potentially leveraging bacteriophages or conjugation, that can efficiently transduce or transfer CRISPR-Cas9 constructs into recalcitrant strains, thus expanding the applicability of this technology [49], [85]. Moreover, optimizing the specificity and minimizing off-target effects of Cas nucleases within the complex P. aeruginosa genome remains a critical area of investigation to ensure precise genetic modifications and prevent unintended cellular perturbations. Further refinement of guide RNA design algorithms and the exploration of novel Cas variants with enhanced fidelity will be crucial for the widespread clinical translation of CRISPR-Cas9-based therapies against P. aeruginosa infections [54]. This includes addressing the immunogenicity of Cas proteins and developing strategies to circumvent host immune

responses, which are paramount for successful in vivo applications. Future research should also focus on integrating CRISPR-Cas9 with other innovative therapeutic strategies, such as phage therapy or antimicrobial peptides, to create synergistic approaches that can overcome the multifaceted resistance mechanisms of P. aeruginosa [17], [27]. This multidisciplinary integration promises to unlock novel approaches for combating multidrug-resistant P. aeruginosa, a critical public health challenge [40], [166]. For instance, CRISPR-Cas systems can be engineered into phages to enhance their lytic activity or to drive selection pressures that restore antibiotic sensitivity in P. aeruginosa [167], [168]. Furthermore, the development of robust delivery methods for CRISPR components into diverse bacterial species, including those with intricate cell envelopes, remains a significant hurdle [64]. This bottleneck underscores the necessity for innovative vector development, potentially leveraging advanced nanotechnology or conjugation mechanisms, to facilitate efficient and broad-spectrum delivery of CRISPR-Cas components [52]. One promising avenue involves the use of engineered nanoparticles as non-viral vectors, which can encapsulate CRISPR-Cas components and bypass bacterial defense mechanisms [169].

CONCLUSION

Scientists delivered a revolutionary treatment method for multidrug-resistant Pseudomonas aeruginosa PAO1 through their development of CRISPR-Cas9 technology. The CRISPR-Cas9 system enables researchers to conduct knockout experiments while using CRISPRi/a for gene expression control and epigenetic regulation to conduct research on how P. aeruginosa controls its pathogenicity and biofilm development and antibiotic resistance mechanisms. The CRISPR-Cas9 system demonstrates its therapeutic potential through its ability to disrupt antibiotic resistance genes which include efflux pump genes and β-lactamase genes and resistance plasmids. Researchers have used CRISPRi to investigate critical genes while controlling their gene expression patterns. The characteristics of CRISPRa make the system applicable for researchers who study the phenotypes that result from gene overexpression experiments.

The clinical use of CRISPR-based therapy for P. aeruginosa infections requires resolution of multiple critical problems before it can become operational. The delivery of CRISPR technology to P. aeruginosa bacteria presents the most significant barrier which hinders effective utilization of the CRISPR system. Researchers have created efficient vectors which include bacteriophages and conjugative plasmids and nanoparticles. The CRISPR system for therapy requires safe implementation which faces challenges from off-target effects and Cas protein immunogenicity. The genetic plasticity of P. aeruginosa allows bacteria to acquire resistance through horizontal gene transfer which creates a second significant problem.

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