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Review Article

DECODING THE MOLECULAR WARFARE: ANTI-CRISPR PROTEINS AS GUARDIANS OF PRECISION IN CRISPR-CAS SYSTEMS

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ABSTRACT: The study explores the discovery, processes, evolution, and uses of anti-CRISPR (Acr) proteins, emphasizing the arms race between phage predation and bacterial defense mechanisms, particularly CRISPR-Cas systems. Acr proteins, identified in *Pseudomonas aeruginosa*, exhibit multifaceted inhibitory actions on CRISPR-Cas systems, disrupting spacer formation, cas protein production, crRNA transcription, and active complex formation. This review categorizes Acr proteins hindering various CRISPR/Cas types and emphasizes their therapeutic potential in treating diseases, manipulating genomes, and refining prokaryotic research. Acr proteins function as on-off switches for regulated gene editing, crucial in improving the safety and accuracy of CRISPR-based therapies. Additionally, the review explores the application of Acr proteins in ecological engineering, specifically in modulating gene drives for responsible and controlled interventions. Acr proteins emerge as crucial tools in understanding and advancing genetic engineering, offering versatile solutions in the ongoing molecular warfare between organisms and their genomic invaders.

Keywords: Anti-CRISPR (Acr) proteins, CRISPR-Cas systems, Ecological engineering, Gene editing, *Pseudomonas aeruginosa*, Therapeutic potential.

INTRODUCTION

The Red Queen hypothesis posits that in the perpetual struggle for survival, organisms must continually adapt and develop new defense mechanisms against parasites to avoid extinction. The co-evolutionary dynamics between bacteria and the viruses that prey on them, called phages, demonstrate this evolutionary dynamic [1]. To protect themselves from phage predation, bacteria use a variety of tactics, such as toxin-antitoxin modules, restriction-modification enzymes, and CRISPR-Cas systems [2]. As a result, phages have developed strategies to get around these defenses, including altering restriction sites and creating compounds that neutralize toxins. Additionally, defense mechanisms against dangerous mobile genetic elements (MGEs), like viruses and plasmids [3], have been established by bacteria and archaea. CRISPR (clustered regularly interspaced short palindromic repeats) and CRISPR-associated (Cas) genes are common defense mechanisms found in 90% of archaea and 50% of bacteria. CRISPR-Cas systems are powerful because they can adapt the foreign DNA segments and develop a particular immunity to them [4]. To subdue these systems, MGEs have created genes encoding CRISPR-Cas inhibitors, or anti-CRISPR (Acr) proteins. The field of Acr protein knowledge has grown significantly, since their discovery in 2013, leading to the identification of 54 different families [5]. The discovery, evolution, functioning mechanisms, and applications of Acrs in the continuing phage-bacteria arms race are all thoroughly covered in this literature.

CLASSIFICATION AND DIVERSITY OF CRISPR-CAS SYSTEMS

CRISPR-Cas systems demonstrate a remarkable capability to adapt to novel invasive mobile genetic elements (MGEs) by integrating short sequences into the CRISPR array. This adaptive process grants

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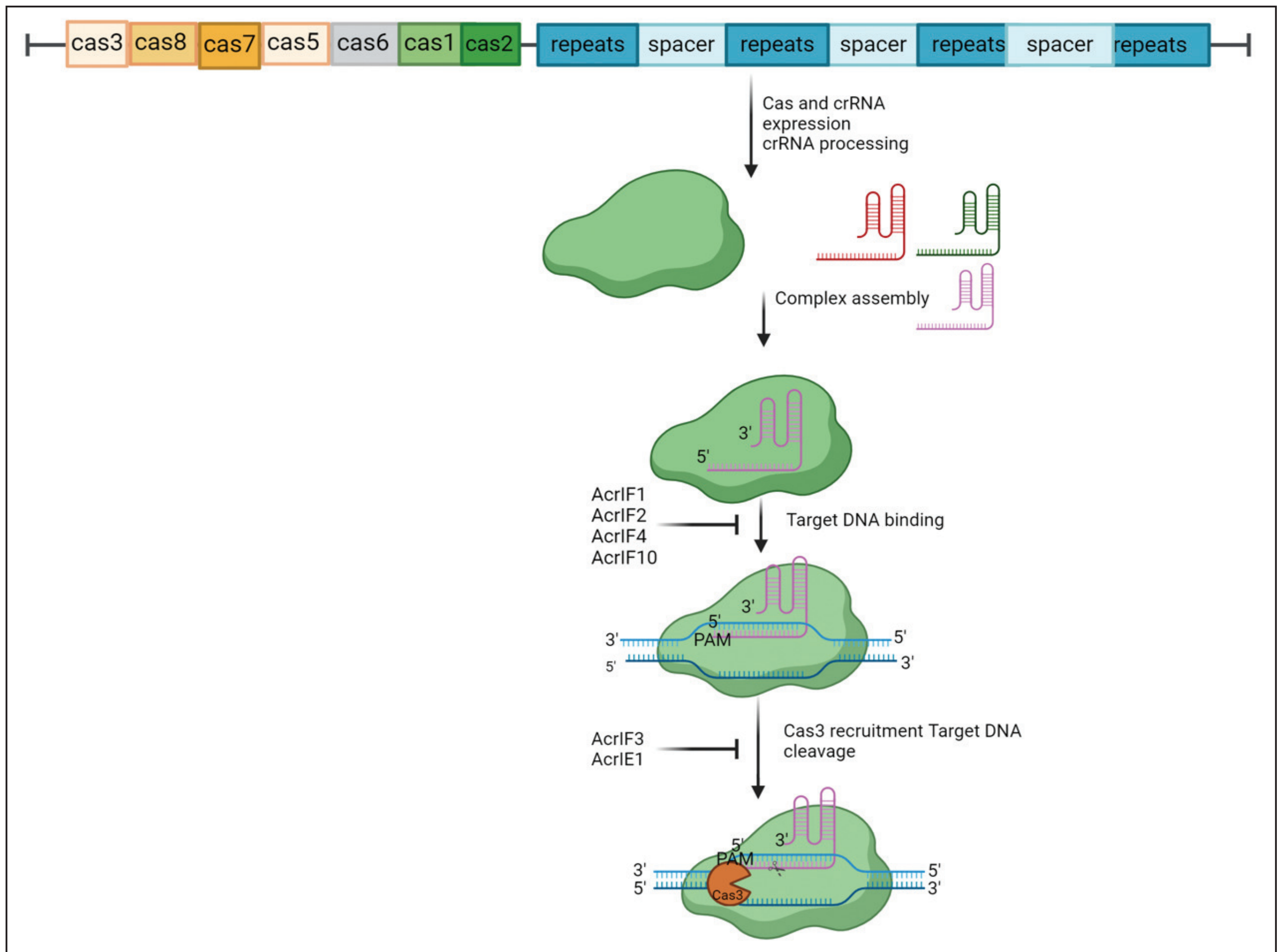

Fig. 1. Anti-CRISPR-Cas activity against Type I CRISPR-Cas system.

immunity against encountered MGEs [2]. Despite shared features, these systems exhibit significant genetic, architectural, and functional variability. Recent categorization attempts have classified the various CRISPR-Cas systems into two classes, six types and over thirty subtypes. Palindromic repeats, spacer lengths, and the distinct complement of Cas proteins are different among these classes [3]. A crRNA complex that includes a minimum of three distinct Cas proteins works together in Class 1 systems (types I, III, and IV) to identify and cleave nucleic acids. In contrast, Class 2 systems (types II, V, and VI) involve a single Cas protein that is attached to crRNA for these purposes, with the well-known CRISPR-Cas9 systems falling under Class 2 and finding extensive use in genome-editing applications [6].

CRISPR loci are made up of two essential components: a collection of Cas genes that encode proteins related to the array and an array with changeable spacers that are mainly obtained from foreign genetic material and are separated by a repeating palindrome [3, 7]. Because of complementarity, the resulting CRISPR-Cas complex allows particular recognition of invasive genetic elements [8]. It consists of crRNA attached to one or more Cas proteins targeted by the spacer, protospacers on foreign DNA are usually surrounded by a protospacer adjacent motif (PAM), which is essential for differentiating self from non-self. PAMs stop CRISPR complexes from attaching themselves to the original arrays [3]. Once bound, the CRISPR-Cas complex makes foreign nucleic acids easier to cleave and destroy. Bacteria can defend themselves against invading nucleic acids with sequence-specificity thanks to CRISPR-Cas systems, thus demonstrating versatility across classes [13]. Class 2 systems use a single effector protein, like Cas9, for genome editing applications, whereas Class 1 systems use multi-subunit Cas protein complexes. Acrs or inhibitors, have been found for Class 1 and Class 2 systems [1].

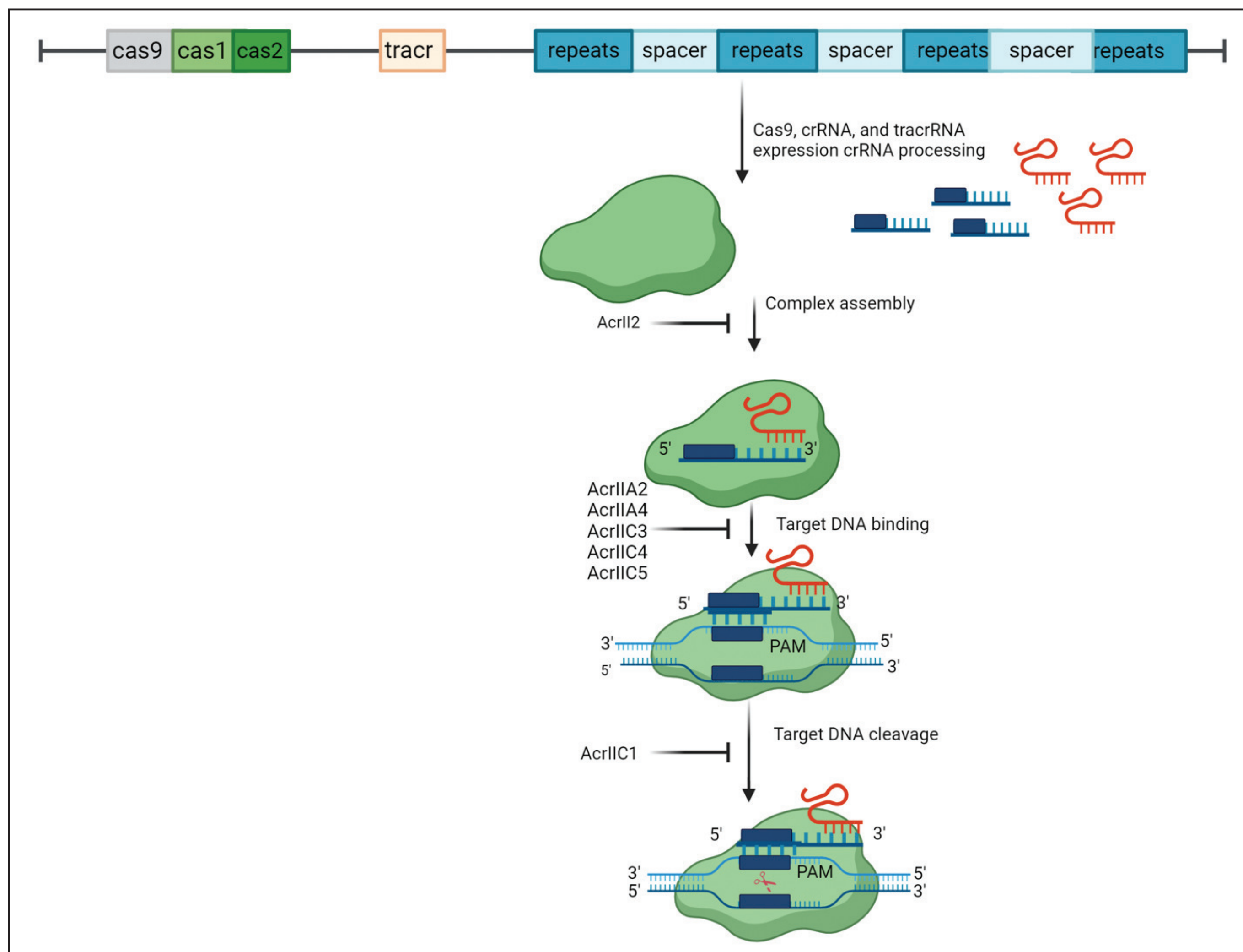

Fig. 2. Anti-CRISPR-Cas activity against Type II CRISPR-Cas system.

ASSESSING RISKS IN CRISPR TECHNOLOGY

Environmental risks

The effects of CRISPR on the environment depend on the selection of CRISPR/Cas9 delivery vectors and host cells. A meticulous assessment of these components is essential to comprehend the nuanced environmental risks associated with the utilization of CRISPR/Cas9 technology [9].

Human health risks

A central concern in the realm of CRISPR/Cas9 technology lies in the risk of off-target genome editing effects. This technology, proficient in inducing site-specific DNA mutations in the human genome, is not without its challenges [10]. Off-target effects, resulting from incomplete homologies between guide RNA (gRNA) and genomic regions, introduce an element of uncertainty. The consequences of these off-target effects remain elusive, with no established prophylactic measures available to counter the unintended nuclear material changes.

Additionally, the risk extends to on-target events, posing potential unintended consequences [11]. The absence of post-exposure preventive strategies leaves individuals exposed to the aftermath of both on- and off-target changes vulnerable. A crucial facet of risk assessment involves considering the target genes, where the selection of hazardous genes, such as oncogenes or tumor suppressors, amplifies the inherent risks associated with CRISPR interventions.

DECODING ANTI-CRISPR MECHANISMS IN PHAGE-BACTERIAL INTERACTIONS

The mystery of how phages effectively infect bacteria even under CRISPR's surveillance came to light with the discovery of anti-CRISPR (Acr) proteins

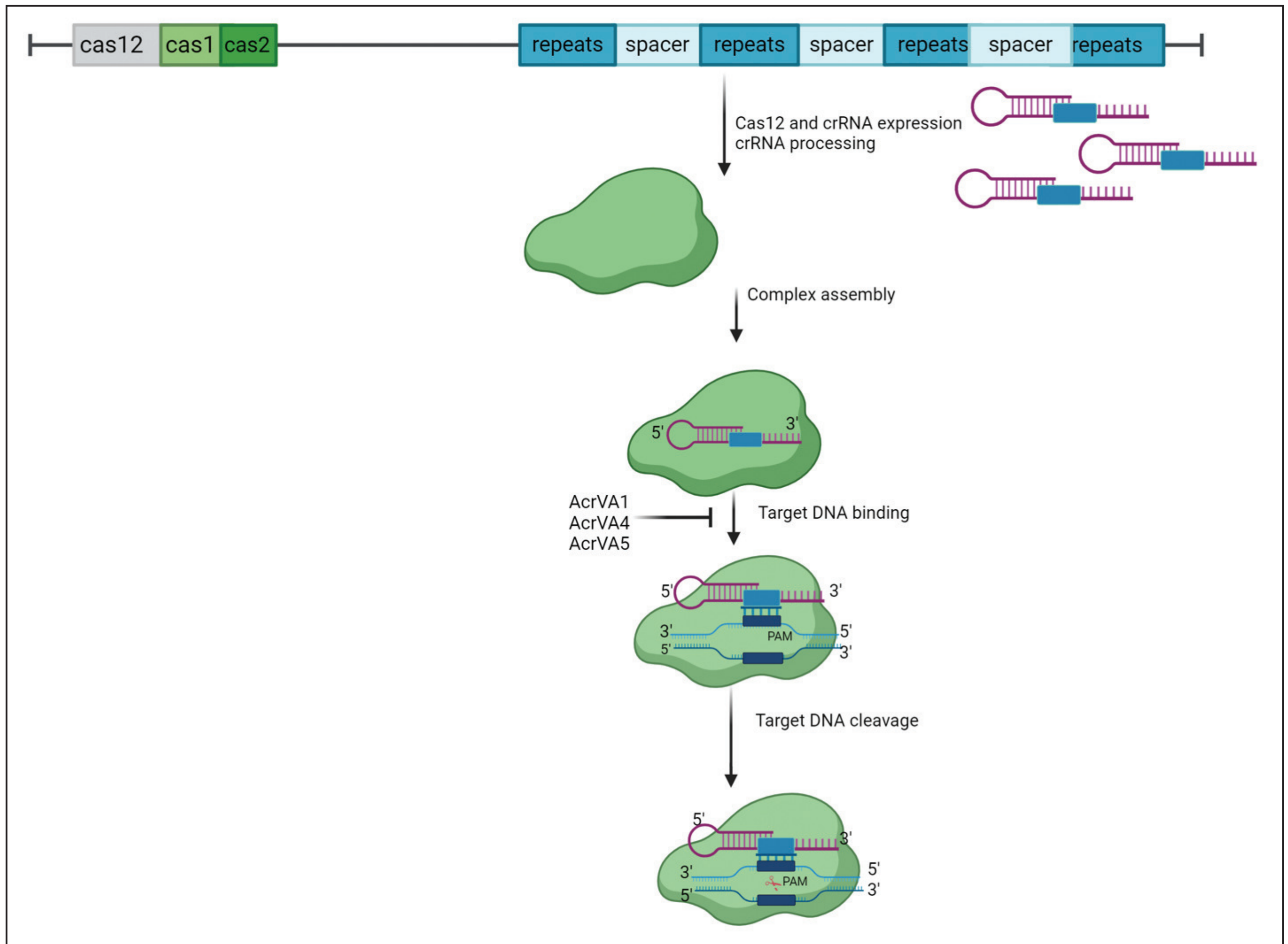

Fig. 3. Anti-CRISPR-Cas activity against Type V CRISPR-Cas system.

in *Pseudomonas aeruginosa*. These proteins have a diverse array of inhibitory actions on the CRISPR-Cas system. They were discovered during an inquiry into the impacts of prophages in *P. aeruginosa*. Acr proteins limit the transcription of crRNA, inhibit binding to foreign DNA elements [6], prevent the formation of active CRISPR-Cas complexes, block the synthesis of new CRISPR spacers, and block Cas protein production [12]. Three prophages were identified through a systematic investigation into prophages that mediate the suppression of the type I-F CRISPR-Cas system through genome comparisons, five different Acr protein families (AcrIF1-AcrIF5) were found to block the type I-F system, while four more families (AcrIE1-AcrIE4) were reported to inhibit the type I-E CRISPR-Cas system in some strains of Pseudomonas algae [13]. Interestingly, every protein in these nine Acr families had a length of less than 140 amino acids and showed no sequence homology to other proteins. This comprehensive exploration highlights the intricate interplay between phages and bacterial defense mechanisms, providing valuable insights into the evolving landscape of CRISPR-Cas system interactions [12].

MECHANISMS OF ANTI-CRISPR INHIBITION IN DIFFERENT CRISPR/CAS TYPES

Type I CRISPR/Cas hindering

Anti-CRISPR (Acr) proteins hinder CRISPR/Cas systems through several distinct stages CRISPR array is shown as a generalized type I CRISPR-Cas locus in the illustrated layout (Fig. 1), together with the expression of cas (CRISPR-associated) genes [14]. Once the transcript of this gene matures, pre-crRNA undergoes maturation to become mature crRNA [15]. The mature crRNA results from the CRISPR/Cas complex along with several Cas proteins. This complex identifies targets, specifically mobile genetic elements (DNA),

through complementary base pairing with the crRNA and recognizing an appropriate PAM sequence [3, 16]. Acr proteins, including AcrIF1, AcrIF2, AcrIF4, and AcrIF10, put a stop to the CRISPR/Cas complex from binding with foreign DNA. The nuclease/helicase (Cas3) that cleaves the DNA target is recruited when the annealing of crRNA results in the creation of an R loop. AcrIF3 and AcrIE1 impede this Cas3 activity [17].

Type II CRISPR/Cas

The Type II CRISPR/Cas system is illustrated (Fig. 2.), where mature crRNA and tracrRNA combine to form a complex with Cas9 [18]. This complex identifies the PAM site and binds to the complementary sequence of crRNA. The loading of Cas9 onto crRNA is hindered by AcrIIC2. AcrIIA2, AcrIIA4, AcrIIC4, and AcrIIC5 restrain the recognition of target DNA [19]. The inhibition of Cas9 by these proteins, such as AcrIIC2 hindering crRNA loading and others like AcrIIA2, AcrIIA4, AcrIIC4, and AcrIIC5 inhibiting target DNA recognition, can potentially be leveraged in cancer research and therapy.

Antagonism against type V CRISPR/Cas

In the Type V CRISPR/Cas system, the surveillance complex comprises expressed, processed, and assembled crRNA along with Cas12. Recognition of target DNA is impeded by AcrVA1, AcrVA4 and AcrVA5 [3]. When the CRISPR/Cas complex initiates binding to complementary target DNA, it starts the nuclease activity of Cas12, which causes a staggered double-stranded DNA break on the target DNA (Fig. 3.).

THERAPEUTIC POTENTIAL OF ANTI-CRISPR PROTEINS IN DISEASE TREATMENT AND GENOME EDITING

Several investigations have indicated that Acr proteins exhibit a broad spectrum of functional activities, enabling researchers to control different characteristics like insertion, deletion, suppression, and single-letter correction. When working with new or anticipated Acrs, it is crucial to determine the major architectural and dynamic elements of Acr-Cas interactions to anticipate inhibitory implications [20]. Exploring these biophysical concepts is therefore of utmost importance. Acrs can treat a wide range of illnesses because of their strong affinity and specificity for CRISPR-Cas systems [21]. This includes diseases brought on by bacteria resistant to drugs, secondary bacterial infections linked to COVID-19 and SARS-CoV, genetic disorders such as Alzheimer's, Parkinson's, and Huntington's diseases, and diseases spread by insects and viruses that can be brought on by controlled genome editing. Treatments based on Acr proteins and their applications in plant science are still in their infancy [22]. To identify common architectural or dynamic aspects of Acr-Cas interactions, it is imperative to investigate the biophysical principles essential for Acr function. With freshly created Acrs, this knowledge can be crucial in forecasting future inhibitory results [6].

EXPANDING APPLICATIONS OF ACR PROTEINS IN PROKARYOTIC

The utilization of Acr proteins in prokaryotic applications within the CRISPR-Cas framework has shown to be a powerful tool. Cas9-based editing, employed across various bacteria from *Escherichia coli* to industrially relevant species like *Clostridium*, *Lactobacillus*, and *Streptomyces*, has demonstrated its efficacy [23]. Acr proteins play a dual role in these applications: firstly, finding the strains where the suppression of endogenous CRISPR-Cas prevents efficient editing and secondly, enhancing temporal control for editing bacterial and phage genomes previously deemed challenging [24]. In the realm of genomic editing and targeted gene repression in bacteria, two approaches exist: introducing an exogenous system or reprogramming an endogenous system. Acr proteins, prevalent in bacteria, can impede the efficiency of these processes, necessitating the identification of anti-CRISPR proteins to enable broader CRISPR-based editing. Beyond identification, Acr proteins can enhance microbial gene-editing strategies, facilitating stable transformation and providing a novel route for engineering viruses [25]. Furthermore, Acr proteins show promise in antibacterial applications by disrupting CRISPR-dependent virulence mechanisms in bacterial pathogens. They also offer the potential to augment phage therapy approaches, countering CRISPR-based phage resistance and improving efficacy against antibiotic-resistant bacteria. The numerous uses for Acr proteins highlight how important they are for developing prokaryotic research and treatment approaches.

PRECISE GENOME EDITING AND GENE REGULATION

The integration of CRISPR-Cas systems into eukaryotic environments, spanning fungi, plants, and mammalian cells, has showcased its versatility. However, challenges arise with the uncontrollable

nuclease activity of Cas enzymes and the variable off-target editing they induce. Current strategies to address these issues have limitations, such as increased size and additional ligands. Delaying the introduction of Acr proteins offers a flexible way to limit off-target editing while using the wild-type Cas enzyme, which is a promising solution [26]. Acr proteins, known for their relatively small size, emerge as potential CRISPR-Cas modulators, especially for *in situ* delivery via adeno-associated viral (AAV) vectors. The controlled regulation of Cas nuclease activity is crucial to mitigate off-target effects, ensuring the efficacy of gene-editing approaches [24]. Successful experiments involving AcrIIA2 and AcrIIA4 delivery in mice, as well as AcrIIC3 inhibition *in vivo*, highlight the promising role of Acr proteins in enhancing CRISPR-Cas applications. Moreover, Acr proteins contribute to the refinement of CRISPR-Cas systems for gene expression modulation without cleavage [27]. By preventing Cas proteins from binding DNA, Acrs aid in spatially and temporally regulating gene activity. Their utility extends to biosensors, synthetic gene circuits, and dissecting off-target events in applications like base editing and recruitment efforts. The diverse applications of Acr proteins underscore their potential in advancing precision genome editing and gene regulation technologies.

CONTROLLING GENE DRIVES FOR RESPONSIBLE ECOLOGICAL ENGINEERING

The potential for ecological engineering has increased with the introduction of CRISPR-Cas9-based technologies, especially through "gene drives" that disperse designed features in populations with previously unheard-of efficiency. Gene drives, which frequently involve a transgenic organism with chromosomally encoded Cas9 [24], have the potential to eradicate invading species, reduce the incidence of insect-borne diseases, and improve the sustainability of agriculture. However, concerns about unforeseen consequences and potential misuse necessitate robust safety measures before employing gene drives in the wild. Acr proteins, which are not affected by the sgRNA sequence, provide a straightforward and adaptable solution to suppress or regulate the strength of gene drive. AcrIIA2 and AcrIIA4 have been shown in recent investigations to be capable of suppressing gene drives, with AcrIIA4 achieving over 99.9% suppression in a yeast model system [28]. The titratable nature of gene drive inhibition, influenced by specific mutations and expression levels of Acr genes, allows for finely tuned control. This presents the potential to use Acr proteins to ensure the safe deployment of gene drive technology, with the ability to halt ongoing drives or reverse their effects. While the efficacy of Acr proteins in animal-based gene drives requires further exploration, their application holds promise for achieving controlled and responsible ecological modifications.

PRECISION CONTROL FOR ENHANCED SAFETY IN CRISPR-BASED THERAPEUTICS

The CRISPR-Cas system has proven to be a powerful tool in genetic engineering, enabling precise DNA cutting and the introduction of desired gene sequences [29]. With CRISPR-based therapeutics advancing to early clinical trials, applications include treating cancer, sickle cell anemia, HIV, and various genetic disorders [30]. However, safety concerns arise from potential unintended edits in prolonged CRISPR system activity, emphasizing the need for regulatory control. In applications such as gene editing, silencing, epigenetic modification, gene drive, imaging, and bacteriophage therapy, anti-CRISPR proteins function as on-off switches, offering precise regulation for CRISPR-Cas and ensuring safety [31]. The development of easily deliverable anti-CRISPR proteins can expedite FDA approval for CRISPR-based drugs, showcasing their pivotal role in therapeutic implementation. This review underscores the significance of anti-CRISPRs as effective inhibitors, shaping the future of CRISPR-based therapeutics [32, 33].

CONCLUSION

In the perpetual struggle between bacteria and viruses, CRISPR-Cas systems have emerged as guardians of genetic integrity, showcasing adaptive immunity against invasive mobile genetic elements. Central to this molecular warfare are anti-CRISPR (Acr) proteins, discovered as potent inhibitors, showcasing diverse mechanisms to block CRISPR-Cas defenses. This review unveils the sophisticated interplay between bacteria and phages, decoding the multifaceted inhibitory actions of Acr proteins. With over 54 distinct families identified, Acrs exhibit intricate strategies, from impeding spacer formation to blocking various stages of CRISPR-Cas machinery. Expanding beyond prokaryotic realms, the review explores the application of Acr proteins in eukaryotic environments. In the burgeoning landscape of CRISPR-based technologies, Acr proteins offer a flexible solution to enhance precision, curbing off-target effects and amplifying

efficacy. Acr proteins play a crucial role in advancing CRISPR-based technologies by enhancing precision, reducing off-target effects, and amplifying efficacy, particularly in eukaryotic environments. By fine-tuning CRISPR activity, Acr proteins provide greater control over genetic modifications, making the technology safer and more effective for applications like gene therapy. This flexible approach not only curbs unintended genetic changes but also expands the potential uses of CRISPR, making it a valuable tool in the evolving landscape of genetic engineering and therapeutic interventions. In the realm of therapeutic interventions, Acr proteins serve as guardians of safety in CRISPR applications. Their role as on-off switches provides a finely tuned control mechanism, crucial for ensuring the safety and regulatory compliance of genetic interventions in clinical settings. As CRISPR-based therapeutics progress, the pivotal role of Acr proteins becomes apparent, guiding the field toward safer and more regulated genetic interventions. This dynamic saga of CRISPR and anti-CRISPR interactions not only illuminates the intricacies of bacterial defense mechanisms but also presents a versatile toolkit for advancing genetic engineering and therapeutic precision. The ongoing exploration promises continuous revelations, unlocking new possibilities and refining our understanding of the dynamic interplay between organisms and their genomic invaders.

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