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REVIEW

Research progress of phage anti-CRISPR proteins

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Abstract The CRISPR-Cas (clustered regularly interspaced short palindromic repeats-CRISPR associated) system, an adaptive immune mechanism in bacteria and archaea, plays a crucial role in defense against phage and plasmid invasion. However, as the main natural enemies of bacteria, bacteriophages have evolved new mechanisms to escape host defenses through rapid mutation and diversification strategies. In response to the CRISPR-Cas system, phages have evolved proteins to inhibit the CRISPR-Cas system, thus revealing the complex evolutionary "arms race" between bacteriophages and their hosts. The study of anti-CRISPR (Acr) proteins offers novel insights into the molecular mechanisms of the CRISPR-Cas system and its potential applications in the precise regulation of gene editing and phage therapy. Consequently, a more profound comprehension of the anti-CRISPR system holds immense potential in numerous research domains. This review systematically summarizes the inhibitory mechanisms of anti-CRISPR proteins, highlights emerging applications, and proposes innovative directions to advance anti-CRISPR research.

Keywords Anti-CRISPR, CRISPR-Cas, Bacteria, Phage, Adaptive immune

INTRODUCTION

The CRISPR-Cas (clustered regularly interspaced short palindromic repeats-CRISPR associated) system represents a genomic defense mechanism widely distributed across prokaryotic organisms, with prevalence observed in nearly 50% of bacterial species and over 90% of archaeal lineages (Makarova *et al.* 2020). Functioning as an adaptive immune system, this molecular apparatus combines CRISPR arrays containing short repetitive sequences with associated Cas proteins to detect and neutralize foreign genetic material from bacteriophages, plasmids, and transposable elements (MGEs) (Barrangou *et al.* 2007; Wang *et al.* 2022). Current classification schemes organize these systems into two operational classes (Classes 1 and 2) and six distinct types (I–VI), with further subdivision into subtypes based on variations in nucleic acid targeting mechanisms (Mohanraju *et al.* 2016). Despite the structural diversity, all CRISPR-Cas variants operate through three conserved functional phases: foreign DNA integration (adaptation), CRISPR RNA biogenesis (expression), and target sequence recognition and cleavage (interference) (Mitić *et al.* 2023) (Fig. 1).

The co-evolutionary dynamics between microbial hosts and viral predators have led to remarkable innovations in molecular countermeasures. Phages employ mechanisms of genetic plasticity, including accelerated mutation rates (approaching 10^{-5} mutations per base per replication cycle) and structural genome reorganization to evade host defenses, such as altering receptor-binding proteins or deploying DNA modification systems (Jiang *et al.* 2024). Bacterial counterstrategies encompass CRISPR-mediated sequence-specific targeting, toxin-antitoxin mediated growth arrest, and population-level protective mechanisms through altruistic cell death (Camara-Wilpert *et al.* 2023). This evolutionary pressure has been selected for viral-encoded counter-defence proteins, exemplified by the 2013 identification of anti-CRISPR (Acr) molecules in *Pseudomonas* phages that directly inhibit Cas protein

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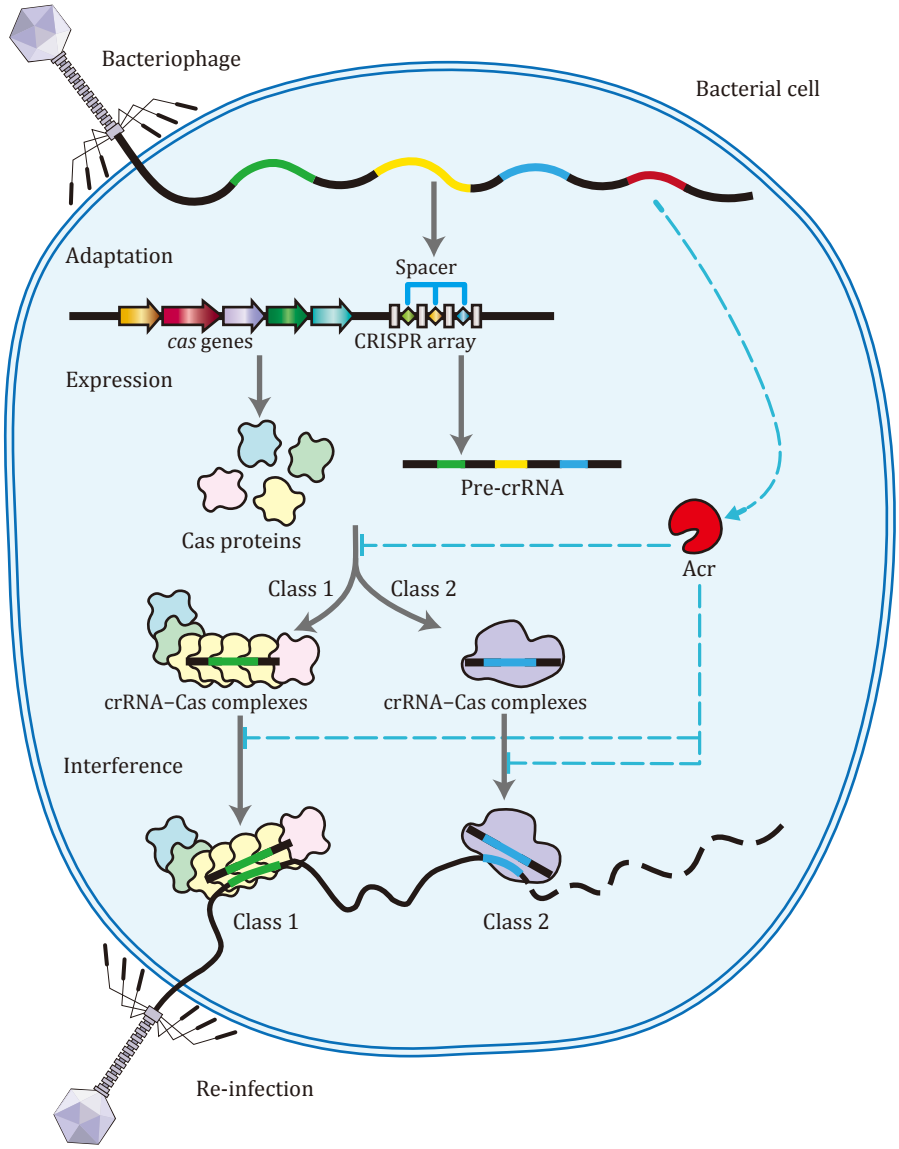

Fig. 1 CRISPR-Cas adaptive immunity in prokaryotes and countermeasures of bacteriophages. Role of CRISPR-Cas system as an adaptive immunity approach in prokaryotes and the counter role of Acrs synthesized from viral genome (blue dashed arrows) to block this genome editing approach

function (Bondy-Denomy *et al.* 2013). Subsequent research has expanded the Acr repertoire to 122 characterized variants, targeting all major CRISPR-Cas types except Type IV (Table 1). These viral inhibitors employ diverse mechanisms, including interference with effector complex formation, competitive DNA binding, and catalytic site occlusion. Beyond their biological significance, Acr proteins (Acrs) have enabled refined CRISPR applications by enhancing editing specificity and improving phage therapy outcomes through controlled Cas9 deactivation.

This review offers an in-depth exploration of the discovery, functional classification, and molecular inhibition mechanisms of Acrs. We also evaluate current detection methods, explore their therapeutic and biotechnological applications, and propose new approaches to better understand the evolutionary dynamics between hosts and pathogens, as well as to improve the accuracy of genome-editing techniques.

HISTORY OF THE DISCOVERY OF ANTI-CRISPR PROTEINS

The search for Acrs began when scientists noticed a contradiction: even with strong CRISPR-Cas immunity,

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Table 1 Important features of two classes of CRISPR-Cas systems and associated anti-CRISPR proteins

Class	Type	Subtype	Effector complex	PAM requirement	Nuclease	Acr proteins	Key references
1	I	I-A	Multisubunit complex	Yes	Cas3	AcrIA1	Bondy-Denomy *et al*. 2013
		I-B				AcrIB1-9	Pawluk *et al*. 2016b
		I-C				AcrIC1-11, AcrVA3	León *et al*. 2021
		I-D			Cas3 and Cas10d	AcrID1	He *et al*. 2018
		I-E			Cas3	AcrIE1-9, AcrIE4-IF7	Bondy-Denomy *et al*. 2015
		I-F				AcrIF1-25, AcrIE4-IF7	Guo *et al*. 2017
	III	III-A		No	Csm3, Csm1	AcrIII-1	Athukoralage *et al*. 2020
		III-B			Cmr4, Cmr2	AcrIIIB1, AcrIII-1	Bhoobalan-Chitty *et al*. 2019
	IV	Unknown	Unknown	Unknown	Unknown	Unknown	/
2	II	II-A	Single protein	Yes	Cas9 RuvC and HNH domains	AcrIIA1-34	Pawluk *et al*. 2014
		II-C				AcrIIC1-9	Lee *et al*. 2018
	V	V-A		Yes	Cas12 RuvC domain	AcrVA1-5	Knott *et al*. 2019b
	VI	VI-A		No	Cas13 HEPN domain	AcrVIA1/2 (*Lse*), AcrVIA1-7(*Lwa*)	Meeske *et al*. 2020
		VI-B				AcrVIB1	Wandera *et al*. 2022

some phages could still infect bacteria. In this context, earlier genomic studies like the 2006 work by Ceyssens *et al.* gained significance. Their analysis of related *Pseudomonas* phage genomes (LKD16 and ΦKMV) revealed small, conserved ORFs missed by prior annotations. The conservation of these putative genes suggested they encoded proteins fulfilling specific functional requirements in the infection process (Ceyssens *et al.* 2006). Following the experimental confirmation of CRISPR-Cas as an adaptive immune system in *Streptococcus thermophiles* (Barrangou *et al.* 2007), researchers observed that certain phages could evade CRISPR defenses through mutations in protospacer adjacent motifs (PAMs) (Deveau *et al.* 2008). These findings solidified the idea that phages might encode proteins capable of actively suppressing CRISPR immunity, formally setting the stage for the systematic exploration that led to the discovery of Acrs.

Prior to 2013, studies not only provided preliminary evidence for the mechanisms of CRISPR inhibition but also laid the crucial methodological groundwork that facilitated the systematic identification of Acrs. Building on these foundational insights, the field has since experienced rapid advancements in the characterization of Acrs (Fig. 2). A landmark achievement was attained in 2013 with the identification of the first Acrs, AcrF1 and AcrF2, encoded by *Pseudomonas aeruginosa* phages (Bondy-Denomy *et al.* 2013). Building directly on the earlier genomic clues, this study combined phage genetic screens with CRISPR activity reporters to demonstrate that AcrF1/F2 specifically inhibits the host's type I-F CRISPR-Cas system. This work not only validated the functional role of the mysterious ORFs but also established a methodological framework for subsequent Acr discovery.

The discovery of Acr proteins entered a phase of accelerated growth following 2013. A pivotal advancement occurred in 2014 when fluorescence-based Cas9 silencing assays enabled the discovery of type II-A inhibitors (AcrIIA2 and AcrIIA4), establishing critical tools for precise spatiotemporal control of CRISPR-Cas9 genome editing (Pawluk *et al.* 2014). The discovery of five novel anti-CRISPR families (AcrF6-AcrF10) in diverse *Proteobacteria*, demonstrating their broad inhibitory activity, was reported in 2016 (Pawluk *et al.* 2016b). Mechanistic studies further revealed diverse

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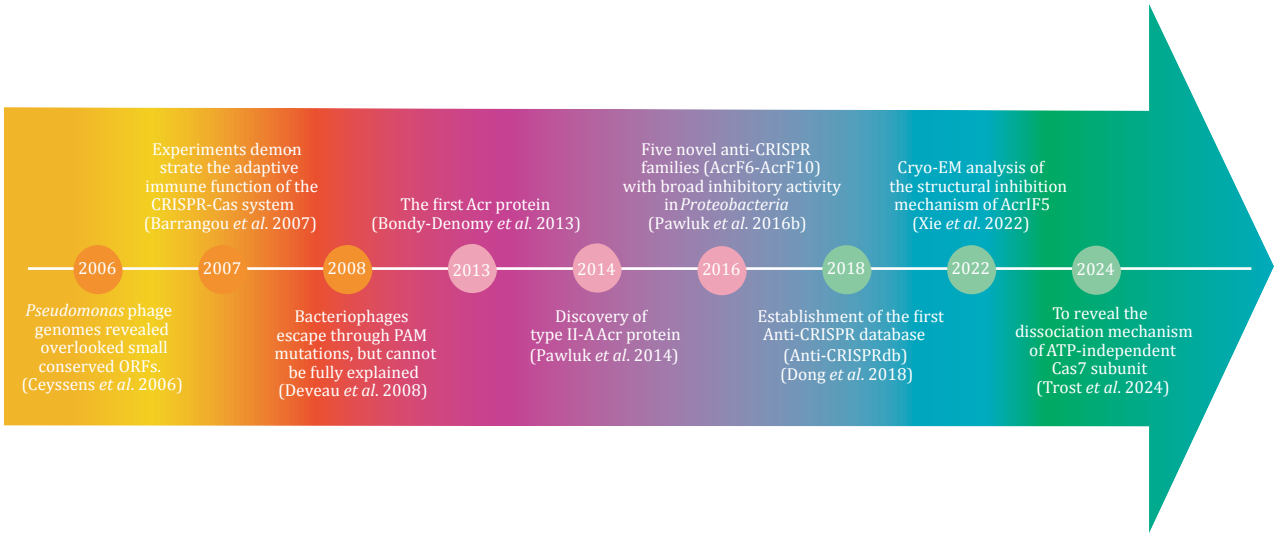

Fig. 2 Timeline of key discoveries in Acr Protein Research. Since the discovery of the first Acr protein in 2013, a series of groundbreaking findings have been reported. In 2018, the launch of the dedicated Anti-CRISPRdb database enabled global classification and standardized knowledge sharing of Acrs. In 2024, a novel mechanism of ATP-independent dissociation of the Cas7 backbone, mediated by AcrIF25, was reported

suppression strategies, including DNA binding interference, enzymatic inhibition, and effector complex destabilization.

Since 2018, anti-CRISPR research has transitioned into a systematic phase characterized by multidisciplinary advances. The establishment of dedicated databases such as Anti-CRISPRdb (Dong *et al.* 2018) has enabled global classification and knowledge sharing of Acrs (Gussow *et al.* 2020; Yin *et al.* 2023). Structural biology approaches, including cryo-electron microscopy, have resolved mechanisms such as AcrIF5's competitive binding to Cas8f helices to block nuclease recruitment (Xie *et al.* 2022) and AcrIF25-mediated ATP-independent dissociation of the Cas7 backbone (Trost *et al.* 2024). Concurrent engineering efforts have optimized Acr functions through structure-guided design, exemplified by enhanced inhibition of Cas9 through targeted mutations in AcrIIA4's binding interface (Li *et al.* 2023). Furthermore, discoveries expanded beyond protein-based inhibitors to include RNA-mediated suppression and small-molecule cyclic nucleotides that disrupt CRISPR signaling pathways (Katz *et al.* 2024; Marino *et al.* 2020), redefining the scope of CRISPR interference mechanisms.

CLASSIFICATION OF ANTI-CRISPR PROTEINS

As an evolutionary arms race strategy, bacteriophages have evolved unique protein inhibitors called Acrs to neutralize the CRISPR-Cas immune defense. Since their groundbreaking discovery in 2013 (Bondy-Denomy *et al.* 2013), 122 different Acrs have been discovered across six distinct CRISPR types (I-VI), with each protein being systematically named to reflect its target specificity and discovery order (Allemailem *et al.* 2024). For instance, AcrIIA4 denotes the fourth Acr found to inhibit type II-A systems (Pawluk *et al.* 2014). This nomenclature system, which was formalized in 2018 (Bondy-Denomy *et al.* 2018), ensures clarity in the classification of Acrs (Table 1). The database (a Google document) can be accessed here: https://tinyurl.com/anti-CRISPR (Bondy-Denomy *et al.* 2018).

CRISPR-Cas systems are categorized into two classes based on effector complex architecture. Class 1 systems (types I, III, IV) employ multi-subunit complexes: type I systems utilize Cascade complexes for crRNA-guided DNA targeting, while type III systems generate cyclic oligoadenylate (cOA) signals to activate nonspecific nucleases (Liu and Doudna 2020). To counteract these defenses, phages encode 58 Acrs in type I systems and two Acrs in type III systems, as summarized in Table 1. No Acrs have been identified for type IV systems, which lack nucleic acid-targeting activity (Bondy-Denomy *et al.* 2018). Class 2 systems (types II, V, VI) rely on a single effector protein: type II Cas9 induces double-strand DNA breaks via HNH and RuvC domains (Hwang and Maxwell 2023; Wen *et al.* 2024); type V Cas12 performs staggered DNA cleavage (Wiegand *et al.* 2020); and type VI Cas13 specializes in RNA targeting (Wandera *et al.* 2022). Corresponding Acrs, such as 43 type II inhibitors, five type V inhibitors, and nine type

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VI inhibitors (Bondy-Denomy *et al.* 2018), demonstrate precise targeting of functional domains (Table 1). Intriguingly, both classes are susceptible to inhibition by bacteriophage-encoded Acrs, which offers unique insights into host-phage coevolution (Sahakyan *et al.* 2023).

The evolutionary origins of Acrs remain enigmatic due to their lack of sequence or structural similarity to known proteins (Wiegand *et al.* 2020). However, advances in computational biology, particularly AlphaFold2 (AF2) (Jumper *et al.* 2021), have enabled high-accuracy predictions of Acr structures, revealing convergent evolution from unrelated protein folds. This structural plasticity allows Acrs to target conserved CRISPR functional domains through divergent mechanisms (An *et al.* 2020).

The functional diversity of Acrs reflects the complexity of their targets. These mechanisms are not confined to preventing complex assembly, blocking DNA binding, or inhibiting enzymatic activity. For instance, AcrVA5 employs a dual strategy: it obstructs Cas12a's DNA-binding groove while recruiting host proteases to degrade the effector (Knott *et al.* 2019b). Similarly, AcrIIIB1 disrupts type III-B immunity by enzymatically degrading cyclic tetra-adenylate (cOA) signaling molecules (Athukoralage *et al.* 2020). Recent studies have revealed even more sophisticated tactics, such as Acr-mediated post-translational modifications (*e.g.*, ADP-ribosylation of Cas3 by AcrIF11 (Niu *et al.* 2020)) and effector dimerization (Jia and Patel 2021).

INHIBITION MECHANISMS OF ANTI-CRISPR PROTEINS

The CRISPR-Cas immune response is comprised of three distinct stages: adaptation (foreign DNA integration), expression (crRNA biogenesis), and interference (target cleavage) (Makarova *et al.* 2020). AcrVA5 inhibits CRISPR-Cas adaptation by directly binding to Cas2, inducing steric hindrance that disrupts Cas1-Cas2-protospacer complex assembly, and enzymatically acetylating Lys55 on Cas2 to abrogate its dsDNA endonuclease activity, thereby blocking protospacer integration and revealing a dual-pronged anti-CRISPR strategy that targets both spacer acquisition and nuclease-dependent viral defense mechanisms (Bi *et al.* 2024). The discovery reveals a novel pathway through which bacteriophages target the CRISPR adaptation stage to escape host immunity. There is currently no clear evidence to suggest that Acr proteins directly act on the expression stage (such as crRNA processing or Cas protein synthesis). However, existing studies suggest that some Acr-related regulatory factors (such as Aca proteins) may indirectly influence gene expression at this stage (Stanley *et al.* 2019).

Acr proteins disrupt interference via four principal strategies (Table 2). Firstly, they block complex assembly by interfering with crRNA processing or effector maturation (*e.g.*, AcrIIC2 trapping apo-Cas9) (Chowdhury *et al.* 2017; Rauch *et al.* 2017). Secondly, they prevent target binding through steric occlusion of nucleic acid interfaces (*e.g.*, Type I AcrIF1) (Guo *et al.* 2017) or structural mimicry (*e.g.*, Type V AcrVA4) (Knott *et al.* 2019a). Thirdly, they inhibit cleavage by catalytic site disruption (*e.g.*, AcrIIC1 inactivating Cas9) (Shin *et al.* 2017) or effector oligomerization (Liu *et al.* 2021). Fourthly, Type III-specific Acrs degrade cyclic oligonucleotide signals (*e.g.*, AcrIII-1 hydrolyzing cA4) (Athukoralage *et al.* 2020), thereby dismantling immune amplification.

Inhibition of CRISPR-Cas complex assembly

Acrs have been shown to disrupt the process of CRISPR-Cas complex assembly, either by blocking the processing of crRNA or its loading, or by acting at the stage preceding the assembly. A prominent example is AcrIIC2, which forms an acidic groove to bind the positively charged bridge helix motif of *Neisseria meningitidis* Cas9 (NmeCas9), preventing sgRNA loading and trapping Cas9 in a protease-sensitive apo state (Thavalingam *et al.* 2019; Zhu *et al.* 2019). This dual mechanism — structural blockade and enhanced proteolysis — enables broad inhibition of diverse Cas9 orthologues, offering biotechnological utility for controlling CRISPR activity (Zhu *et al.* 2019). Similarly, AcrIIA1 has been observed to bind the conserved His840 residue in the HNH domain of apo-Cas9 or sgRNA-bound Cas9, thereby triggering Cas9 degradation *in vivo* through a lysogenic pathway (Osuna *et al.* 2020a). Further studies revealed that Phe115 in AcrIIA1's unstructured C-terminal loop is critical for this interaction, though degradation requires host-specific factors absent *in vitro* (Osuna *et al.* 2020a). Strikingly, AcrIIA1 functions as a dimeric bifunctional inhibitor, with its C-terminal domain suppressing Cas9 activity, and its N-terminal HTH motif repressing its own transcription. This enables the dynamic tuning of expression to match Cas9 levels (Osuna *et al.* 2020b).

Anti-CRISPR proteins employ stage-specific strategies to block Cas9 activity: AcrIIA15 (like AcrIIA1) prevents Cas9-sgRNA complex assembly by steric hindrance (Watters *et al.* 2020). AcrIIA15 targets the early stage of Cas9 activity (complex assembly), while AcrIIA13/AcrIIA14 interfere with Cas9 function at later

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Table 2 Four anti-CRISPR strategies for different functional stages

Action stage	Class 1		Class 2		
	Type I	Type III	Type II	Type V	Type VI
Inhibition of complex assembly	/	/	AcrIIC2[1] AcrIIA1[2] AcrIIA15[3]	/	AcrVIA1[4]
Blocking target binding	AcrIF1/8/9/14[5] AcrIF2/6/7/10/11[6]	/	AcrIIA2/4/6[7] AcrIIA13[8]	AcrVA1/5[9] AcrVA4[8]	/
Prevention of cleavage	AcrIC1[10] AcrIF3[11] AcrIE1/D1[12]	/	AcrIIC1/3[13] AcrIIA14[3]	/	/
Degradation of signalling molecules	/	AcrIIIB1[14] AcrIII-1[15]	/	/	/

References: 1. Thavalingam *et al*. 2019; 2. Osuna *et al*. 2020a; 3. Watters *et al*. 2020; 4. Meeske *et al*. 2020; 5. Guo *et al*. 2017; 6. León *et al*. 2021; 7. Dong *et al*. 2017; 8. Peng *et al*. 2019; 9. Knott *et al*. 2019b; 10. Wang *et al*. 2016b; 11. Bondy-Denomy *et al*. 2015; 12. Pawluk *et al*. 2017; 13. Harrington *et al*. 2017; 14. Bhoobalan-Chitty *et al*. 2019; 15. Athukoralage *et al*. 2020

stages (DNA binding or DNA cleavage) (Kim *et al*. 2019; Watters *et al*. 2020) and enforce Cas9 dimerization to lock it in a cleavage-inactive conformation post-DNA binding (Harrington *et al*. 2017; Zhu *et al*. 2019). All three proteins exhibit dual functionality, coupling Cas9 suppression with autoregulatory transcriptional control (Watters *et al*. 2020), balancing immune evasion and host fitness. This multi-layered targeting — spanning RNP assembly, DNA engagement, and catalytic activation — highlights the evolutionary sophistication of anti-CRISPR systems.

Type VI anti-CRISPR proteins employ precise molecular mechanisms to suppress Cas13-mediated immunity. In Type VI CRISPR-Cas systems, crRNA-guided Cas13 recognizes viral RNA and subsequently activates promiscuous RNA cleavage (Liu *et al*. 2017; Meeske *et al*. 2019). AcrVIA1, encoded by the φLS46 listeriophage, subverts this process by binding directly to the guide-exposed face of Cas13a, simultaneously engaging both crRNA and key structural domains (helical 1, N-terminal, HEPN-2, and linker domains) (Meeske *et al*. 2020). This dual interaction sterically blocks target RNA access to the crRNA and prevents the conformational rearrangement required to align the HEPN nuclease domains for activation. Mutational studies identify critical AcrVIA1 residues (*e.g*., Tyr39, Ser40, Asn43) and structural elements (C-terminal helices) essential for inhibition (Meeske *et al*. 2020), underscoring the precision of its steric and allosteric blockade.

Blocking of target DNA binding

Anti-CRISPR (Acr) proteins employ diverse strategies to block target DNA binding at distinct molecular stages, effectively neutralizing CRISPR-Cas immunity. In Type I systems, Acrs primarily interfere with two critical steps: crRNA-DNA hybridization and protospacer adjacent motif (PAM) recognition. For instance, AcrIF1, AcrIF8, AcrIF9, and AcrIF14 sterically hinder crRNA-guided DNA pairing by binding to Cas7 subunits within the Cascade complex. Cryo-EM studies reveal that AcrIF1 induces localized conformational changes in Cas7, creating physical barriers to DNA access and reducing DNA-binding affinity by over 90% in biochemical assays (León *et al.* 2021; Wang *et al.* 2021; Yang *et al.* 2021). Similarly, AcrIF9 triggers nonspecific DNA binding, sequestering the Cascade complex away from genuine targets (Hirschi *et al.* 2020; Wang *et al.* 2023; Zhang *et al.* 2020). Parallel strategies target PAM recognition — a prerequisite for DNA engagement. AcrIF2, AcrIF6, AcrIF7, and AcrIF10 bind the conserved "K-wedge" loop in Cas8, directly occluding PAM interaction (León *et al.* 2021; O'Brien *et al.* 2020; Tuminauskaite *et al.* 2020). Notably, AcrIF11 adopts a unique enzymatic inactivation strategy, mono-ADP-ribosylating Cas8's Asn250 to irreversibly disrupt PAM recognition (Shibata *et al.* 2020), contrasting with reversible steric blockers. Beyond direct DNA exclusion, AcrIF25 dismantles the Cascade complex through energy-independent disassembly, sequentially stripping Cas7 subunits by exploiting weak interfacial interactions between them — a rare example of passive macromolecular disassembly (Trost *et al.* 2024).

In Type II systems, where DNA recognition hinges on a single Cas9 effector, Acrs demonstrate analogous targeting logic but adapt to structural singularities. AcrIIA2 and AcrIIA4 exploit conformational changes induced by sgRNA loading, binding with high affinity to

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the Cas9-sgRNA complex (Jiang *et al.* 2019, 2021; Kim *et al.* 2018; Liu *et al.* 2019). AcrIIA2 occupies the PAM-interacting (PI) site, thereby locking the HNH nuclease domain and blocking dsDNA binding (Jiang *et al.* 2019; Liu *et al.* 2019), while AcrIIA4 additionally obstructs the RuvC domain's access to the nontarget DNA strand (Kim *et al.* 2018; Shin *et al.* 2017). In contrast to these mechanisms, AcrIIA6 exerts a subtler mode of action, which disrupts the allosteric coupling between PAM recognition and DNA unwinding, indirectly reducing DNA-binding affinity (Fuchsbauer *et al.* 2019). These variations highlight a functional continuum in Acr strategies — ranging from direct competitive binding to allosteric interference — converging on the critical step of target DNA engagement. AcrIIC4 and AcrIIC5 inhibit Cas9 activity by blocking DNA binding, likely through structural interference that prevents PAM recognition or R-loop stabilization (Lee *et al.* 2018). AcrIIA13 inhibits the binding of Cas9 to target DNA, directly blocking DNA cleavage activity and leading to the failure of genome editing functionality (Watters *et al.* 2020).

In Type V CRISPR-Cas systems, Acr proteins deploy diverse strategies to counteract Cas12a through enzymatic inactivation and structural interference. AcrVA1 operates via a dual mechanism where one molecule binds the PAM-interacting domain of the Cas12a–crRNA complex, sterically hindering PAM recognition while its catalytic triad (Arg41, His42, His45) cleaves the crRNA to irreversibly disable the complex (Knott *et al.* 2019b; Marino *et al.* 2018; Watters *et al.* 2018; Zhang *et al.* 2019). A secondary AcrVA1 molecule concurrently obstructs the DNA-binding channel, preventing crRNA-DNA hybridization and ensuring robust suppression. In contrast, AcrVA5 exhibits enzymatic precision by acetylating Lys635, a critical residue for PAM recognition in Cas12a (Dong *et al.* 2019). Structural analyses confirm this acetyltransferase activity introduces steric clashes that disrupt DNA binding, with mutational studies (*e.g.*, K635R in Cas12a or acetyl-CoA-binding site mutations in AcrVA5) abolishing inhibitory effects. AcrVA4 employs multi-step inhibition by binding the Cas12a–crRNA complex through electrostatic mimicry of pre-crRNA via acidic residues (Glu184/Glu204), anchoring the crRNA pseudoknot (Knott *et al.* 2019a; Zhang *et al.* 2019). Cryo-EM studies reveal that its monomeric C-terminal domain alone is capable of preventing crRNA–DNA duplex formation and triggering premature DNA dissociation, even post-target cleavage (Peng *et al.* 2019). Collectively, these findings underscore the adaptability of Type V Acrs in disrupting DNA recognition through covalent modification, catalytic RNA degradation, and competitive spatial obstruction, emphasizing their evolutionary refinement to target both functional and structural vulnerabilities in Cas12a.

Prevention of target DNA cleavage

Acrs prevent DNA cleavage through diverse mechanisms targeting nuclease activation. In Type I systems, AcrIC1 inhibits the Cas3 nuclease by binding to its HD domain, blocking recruitment to the Cascade complex (León *et al*. 2021). AcrIF3 competitively blocks Cas3 recruitment to the Cascade complex by structurally mimicking the Cas8 C-terminal domain, which normally mediates this interaction (Bondy-Denomy *et al*. 2015; Chowdhury *et al*. 2017). Structural studies reveal that AcrIF3 traps Cas3 in an ADP-bound state, obstructing its DNA entry tunnel and halting cleavage (Wang *et al*. 2016a, b). Similarly, AcrIE1 dimerizes with Cas3, disabling its helicase-nuclease activity via C-terminal interactions (Pawluk *et al*. 2017), while AcrIC3 uniquely disrupts the Cas3–Cas8 interface by binding the Cas3 C-terminal domain, as evidenced by the escape of a Cas3–Cas8 fusion protein from inhibition (León *et al*. 2021). These strategies highlight convergent evolution in blocking Cas3 activation across Type I subtypes.

In Type I-D systems, AcrID1, an anti-CRISPR protein encoded by archaeal viruses such as SIRV2 and SIRV3, specifically inhibits the type I-D CRISPR-Cas system by directly binding to the large subunit Cas10d of the effector complex. Structural studies reveal that AcrID1 forms a dimer that sterically blocks the Cas10d PAM-recognition domain (PRD) and its HD nuclease active site, preventing dsDNA engagement and subsequent recruitment of the Cas3 helicase required for DNA cleavage (He *et al*. 2018; Manav *et al*. 2020). This competitive inhibition disrupts the conformational rearrangements essential for R-loop stabilization and target strand degradation (Schwartz *et al*. 2022). Beyond its role in immune evasion, AcrID1 has been repurposed as a selectable marker for CRISPR-based genome editing of archaeal viruses, enabling targeted gene knockout (Mayo-Muñoz *et al*. 2018).

Structural analysis reveals that AcrIIC1 sterically occludes the catalytic center of the HNH domain of Cas9 (Harrington *et al.* 2017; Jinek *et al.* 2012). AcrIIC3 enhances suppression by inducing Cas9 dimerization, which mispositions the HNH and RuvC domains, blocking both DNA strand cleavages (Kim *et al.* 2019; Sun *et al.* 2019; Zhu *et al.* 2019). Similarly, AcrIIA14 does not interfere with Cas9 binding to target DNA, but instead triggers Cas9 dimerization, forcing it into an inactive conformation, similar to the allosteric inhibition mechanism of AcrIIC3 (Watters *et al.* 2020).

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Degradation of cyclic oligonucleotide signalling molecules

The Type III CRISPR-Cas system amplifies immune responses by synthesizing cyclic oligoadenylate (cOA) second messengers (*e.g.*, cA4). For example, upon detecting viral RNA, the Csm/Cmr effector complex activates the Palm domain of Cas10 to generate cOA from ATP, which allosterically activates nonspecific RNases like Csm6/Csx1 for RNA degradation (Foster *et al.* 2019; Jia *et al.* 2019a; Niewoehner *et al.* 2017; Rostøl and Marraffini 2019). Alternatively, to mitigate self-toxicity, endogenous cyclic nucleases within Csm6/Csx1 hydrolyze cOA into linear fragments (*e.g.*, cA4→ApA>p) to terminate RNase activity (Garcia-Doval *et al.* 2020; Jia *et al.* 2019b; Molina *et al.* 2019; Smalakyte *et al.* 2020).

Viruses employ two strategies to disrupt this pathway: blocking cOA production or degrading signaling molecules. For example, AcrIIIB1 binds the subtype III-B Cmr complex, sterically obstructing the Cas10 Palm domain's ATP-binding pocket and preventing cOA synthesis (Bhoobalan-Chitty *et al.* 2019). In contrast, AcrIII-1 directly hydrolyzes cA4 via a catalytic histidine (His47), decoupling immune signaling from effector activation. Structural studies reveal AcrIII-1's mobile loop envelops cA4, positioning His47 to cleave the cyclic phosphate linkage — a mechanism crippled in H47A mutants, which exhibit a 2,500-fold reduction in activity (Athukoralage *et al.* 2020).

ADVANCEMENTS IN ANTI-CRISPR RESEARCH

The study of anti-CRISPR (Acr) proteins has undergone transformative methodological progress, spanning discovery platforms to mechanistic analyses. Early studies relied on phenotype-based screening strategies derived from host-phage interactions. Initial identification strategies focused on phage-host coevolution dynamics, exemplified by Bondy-Denomy *et al.*'s seminal discovery of the AcrF1-F5 family through genomic analysis of CRISPR-evading *Pseudomonas aeruginosa* phages (Bondy-Denomy *et al.* 2013). While this approach preserved ecological relevance, its reliance on cultivable phage-host pairs restricted throughput and generalizability. Subsequent developments introduced self-targeting CRISPR systems that bypassed phage dependency, as demonstrated by Marino *et al.*, who identified AcrIIA23 through targeted inactivation of essential *Escherichia coli* genes (Marino *et al.* 2018). These host-centric screens improved scalability but remained constrained by the availability of endogenous CRISPR systems.

Genomic innovations expanded discovery horizons through comparative analysis of anti-defense gene clusters. Pawluk *et al.* pioneered this approach by identifying AcrIIA4 through the analysis of conserved synteny patterns in the vicinity of known defense genes in microbial genomes (Pawluk *et al.* 2016b). While this method is cost-efficient for mined genomes, its predictive power depends on existing functional annotations. The integration of machine learning with metagenomic datasets represented a paradigm shift, as exemplified by Doron *et al.*'s AcrRanker algorithm, which enabled systematic Acr prediction in uncultivated microbiota through sequence feature prioritization (Doron *et al.* 2018). Contemporary pipelines now combine predictive tools such as CRISPRCasTyper (Russel *et al.* 2020) with structural prediction platforms such as AlphaFold2 (Jumper *et al.* 2021), though the predictive accuracy of these tools remains contingent on the quality of the training data and the evolutionary conservation.

Functional validation has evolved into a multi-tiered framework. Phage plaque assays provide phenotypic evidence, as demonstrated by Hynes *et al.*, who showed that AcrIIA6 can restore phage infectivity in CRISPR-immune *Streptococcus thermophilus* from 0.01% to near-complete surviva l (Hynes *et al.* 2018). Structural analyses complement these findings by revealing mechanistic bases, with the AcrIIA6 dimer physically obstructing the Cas9's PAM-interaction domain through helix-turn-helix motifs (Hynes *et al.* 2018). Biochemical approaches further decode inhibitory specificity, as shown by Dong *et al.* (2019) where AcrVA5 abolished Cas12a activity at substoichiometric ratios (1:2 AcrVA5:Cas12a) via Lys635 acetylation. Evolutionary validation through residue-specific mutagenesis (K635R resistance mutation) confirmed functional conservation (Dong *et al.* 2019). This multifaceted methodology, encompassing phenotypic rescue, structural interference, enzymatic suppression, and evolutionary conservation, establishes a rigorous paradigm for CRISPR component validation.

The advancement of mechanistic understanding of anti-CRISPR proteins has been achieved through the application of convergent methodology. Dong *et al.* employed X-ray crystallography to demonstrate that AcrIIA4 structurally mimics PAM-containing dsDNA, competitively occluding the Cas9-substrate interface (Dong *et al.* 2017). Functional validation revealed near-stoichiometric inhibition — at 1:1 molar ratio relative to 10 nmol/L Cas9–sgRNA, AcrIIA4 suppressed >90% DNA cleavage activity, consistent with sub-10 nmol/L effective affinity. This dual evidence of molecular

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mimicry and potent inactivation establishes AcrIIA4 as a Cas9-specific failsafe (Dong *et al.* 2017; Shin *et al.* 2017). Building on these structural insights, molecular dynamics simulations delineated the temporal trajectory of AcrIIA4-induced conformational locking, wherein Cas9's catalytic domain is stabilized in a DNA-inaccessible state (Yang and Patel 2017). The synergy of structural biology, quantitative biochemistry, and computational modeling exemplifies a paradigm for deconstructing CRISPR inhibition mechanisms.

APPLICATIONS OF ACR PROTEINS

The discovery of Acrs has not only deepened our understanding of host-phage coevolution mechanisms but also provided revolutionary tools for fields such as biotechnology, gene therapy, and synthetic biology (Table 3). For example, AcrIIA4 reveals its conformational activation pathway by blocking the HNH and RuvC domains of Cas9 (Dong *et al.* 2017), while AcrVA1 elucidates its target recognition mechanism by occupying the DNA-binding channel of Cas12a (Yang and Patel 2017). These findings contribute to a more profound comprehension of the CRISPR system and lay the foundation for the development of controllable gene-editing tools (Pawluk *et al.* 2016a). In the field of biotechnology, Acrs have been used for gene editing regulation (Marino *et al.* 2020), phage therapy synergistic effects (Gordillo Altamirano and Barr 2021), and other applications.

Table 3 The key application areas and representative cases of anti-CRISPR proteins (Acr)

Application domain	Specific use	Mechanism	Representative Acr proteins	References
Gene editing control	Spatiotemporal Cas9 regulation	miRNA-responsive Acr expression for tissue-specific inhibition	AcrIIC3	Lee *et al*. 2019
	Reducing CRISPR off-targets	Blocking Cas9-DNA binding or catalytic activity to minimize nonspecific cleavage	AcrIIA4, AcrVA1	Dong *et al*. 2017; Zhang *et al*. 2019
Phage therapy	Enhancing phage infectivity	Suppressing host CRISPR-Cas systems to expand phage host range	AcrIIA family	Gordillo Altamirano and Barr 2021
	Multi-target combinatorial therapy	Combining Acrs targeting diverse CRISPR types for broad-spectrum phage cocktails	AcrVA5, AcrIII-1	Knott *et al*. 2019b; Athukoralage *et al*. 2020
Synthetic biology	Dynamic gene circuit design	Reversible CRISPR-Cas control via inducible Acr expression systems	AcrIIA4	Pawluk *et al*. 2016a
Basic research tools	Mechanistic studies of CRISPR	Inhibiting specific Cas functions to probe conformational changes or active sites	AcrIIA4, AcrIF1	Guo *et al*. 2017; Yang and Patel 2017
	Host-phage coevolution analysis	Using Acr escape mutants to study CRISPR resistance evolution	AcrF1/F2	Bondy-Denomy *et al*. 2013
Biocontainment	Safeguarding gene drives	Acr as a "kill switch" to limit uncontrolled CRISPR propagation in ecosystems	AcrIIC1	Marino *et al*. 2020

The off-target effects of the CRISPR-Cas9 system remain a major safety challenge limiting its clinical application, particularly during systemic delivery (*e.g*., via AAV vectors), where nonspecific editing in non-target tissues may lead to genomic damage risks. To precisely regulate Cas9 activity, Lee *et al*. proposed a strategy utilizing microRNA (miRNA)-regulated anti-CRISPR (Acr) proteins to achieve spatial control of editing through tissue-specific Cas9 inhibition (Lee *et al.* 2019). In this study, the coding gene for AcrIIC3 (specifically inhibiting Nme2Cas9) was fused with binding sites for miR-122, a miRNA highly expressed in liver tissue, to construct a miRNA-responsive Acr expression system (Lee *et al.* 2019). In the liver, miR-122 mediates the degradation of Acr mRNA, lifting its suppression of Cas9 and enabling targeted genome editing. Conversely, in non-target tissues lacking miR-122 (*e.g*., the heart), sustained Acr expression completely blocks Cas9 activity, significantly reducing off-target editing frequencies (5.2% editing efficiency in the liver versus 0.33% in the heart) (Lee *et al.* 2019). By co-delivering Nme2Cas9, sgRNA, and

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miR-122-responsive Acr via AAV9 vectors, this strategy successfully achieved liver-specific editing in a mouse model while suppressing off-target events in non-target tissues such as the heart to background levels (Lee *et al.* 2019). This approach not only validates the efficacy of Acrs in spatiotemporal control of CRISPR activity but also provides a critical safety optimization framework for systemic gene therapy, particularly suited for clinical scenarios requiring strict editing confinement (*e.g.*, genetic disorders or cancer treatment). Future adaptations, such as replacing miRNA-responsive elements to target other tissues (*e.g.*, miR-1 for cardiac tissue or miR-133 for muscle), could further enhance the versatility of this platform, offering a universal tool for precision medicine.

Anti-CRISPR (Acr) proteins, which are the products of the evolutionary arms race between bacteria and bacteriophages, have emerged as groundbreaking tools for phage therapy, particularly with regard to overcoming host range limitations and enhancing infection efficiency. Bacteria employ CRISPR-Cas systems to recognize and cleave invading phage genomes, forming a natural immune barrier that severely restricts the therapeutic potential of phages. Acrs counteract this defense by specifically inhibiting the activity of CRISPR-Cas complexes, either by blocking DNA binding (Yang and Patel 2017) or by interfering with nuclease function (Dong *et al.* 2017). This effectively dismantles the protective mechanism and significantly expands the phage host range. Furthermore, the introduction of Acrs delays bacterial immune clearance of phages, prolonging phage replication time within the host and thereby boosting lysis efficiency. Notably, the diversity of Acrs (*e.g.*, the AcrIIA and AcrIIC families targeting distinct Cas subtypes) provides a molecular foundation for designing broad-spectrum phage cocktail therapies while minimizing the likelihood of bacterial escape through single mutations. However, the application of Acrs requires careful consideration of their potential nonspecific effects on host microbiota and immunogenicity. Future optimization through engineering strategies — such as tissue-specific expression or nanocarrier-targeted delivery — will further refine their precision and safety, paving the way for safer and more effective phage-based therapeutic interventions.

DISCUSSION

The discovery and characterization of anti-CRISPR (Acr) proteins have profoundly advanced our understanding of the evolutionary arms race between phages and their microbial hosts, while simultaneously catalyzing breakthroughs in both fundamental CRISPR biology and applied biotechnology. At their core, Acrs act as molecular probes, offering unprecedented insights into the conformational dynamics, substrate recognition, and regulatory mechanisms of Cas nucleases. These mechanistic insights not only deepen our comprehension of CRISPR–Cas systems but also provide a blueprint for engineering next-generation gene-editing tools with enhanced precision.

Technologically, Acrs have emerged as versatile tools for applications spanning precision genome editing, phage therapy, and synthetic biocontainment. In therapeutic contexts, Acr-mediated control of CRISPR activity — such as tissue-specific Cas9 inhibition via miRNA-responsive Acr systems — has mitigated off-target risks, enabling safer *in vivo* gene therapies. Meanwhile, Acr-enhanced phages have shown promise in overcoming bacterial CRISPR defenses, offering a novel strategy to combat antibiotic-resistant pathogens. However, challenges persist. Delivery inefficiency, potential off-target effects in non-host environments, and unresolved ecological risks — such as unintended horizontal gene transfer — demand rigorous optimization. Addressing these hurdles will require interdisciplinary collaboration, integrating structural biology, computational modeling, and bioengineering. For example, machine learning-guided Acr design and nanoparticle-based delivery platforms could enhance specificity and efficacy.

Looking ahead, the full potential of Acrs hinges on bidirectional integration of discovery and translation. Fundamental studies, such as *in situ* mechanistic analyses of Acr-Cas interactions and metagenomic surveys of novel Acr families, will expand the molecular toolkit. Concurrently, applied innovations — like CRISPR-Acr toggle switches for dynamic gene regulation or Acr-phage cocktails for precision antimicrobial therapy — must prioritize ethical and ecological safeguards. By bridging these realms, Acr research promises to deliver sustainable solutions to global challenges, from curbing antimicrobial resistance to enabling controllable genome therapies. Ultimately, this synergy between basic science and technological ingenuity will define the next frontier in CRISPR-Acr applications, ensuring their responsible deployment across biomedical and environmental landscapes.

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Compliance with Ethical Standards

Conflict of interest Mengwen Zhao, Ang Gao and Yalan Zhu declare that they have no conflict of interest.

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Human and animal rights and informed consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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