Anti-CRISPRs: Protein Inhibitors of CRISPR-Cas Systems

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Abstract

Clustered regularly interspaced short palindromic repeats (CRISPR) together with their accompanying *cas* (CRISPR-associated) genes are found frequently in bacteria and archaea, serving to defend against invading foreign DNA, such as viral genomes. CRISPR-Cas systems provide a uniquely powerful defense because they can adapt to newly encountered genomes. The adaptive ability of these systems has been exploited, leading to their development as highly effective tools for genome editing. The widespread use of CRISPR-Cas systems has driven a need for methods to control their activity. This review focuses on anti-CRISPRs (Acrs), proteins produced by viruses and other mobile genetic elements that can potently inhibit CRISPR-Cas systems. Discovered in 2013, there are now 54 distinct families of these

proteins described, and the functional mechanisms of more than a dozen have been characterized in molecular detail. The investigation of Acrs is leading to a variety of practical applications and is providing exciting new insight into the biology of CRISPR-Cas systems.

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INTRODUCTION

Bacteria and archaea have developed many systems to defend against potentially harmful mobile genetic elements (MGEs), such as viruses and plasmids (1, 2). Clustered regularly interspaced short palindromic repeats (CRISPR) and their accompanying *cas* (CRISPR-associated) genes comprise one of the most prevalent of these systems, occurring in approximately 50% of bacteria and 90% of archaea (3). CRISPR-Cas systems present a particularly formidable defense because they are adaptive, acquiring specific immunity to segments of foreign DNA after exposure to these elements (for reviews see 4, 5). To combat these potent systems, MGEs have acquired genes encoding inhibitors of CRISPR-Cas systems, known as anti-CRISPR (Acr) proteins. First discovered in 2013 (6), knowledge of these proteins has rapidly expanded, with 54 distinct families currently recognized (7). In this review, we describe the discovery, functional mechanisms, evolution, and applications of Acrs.
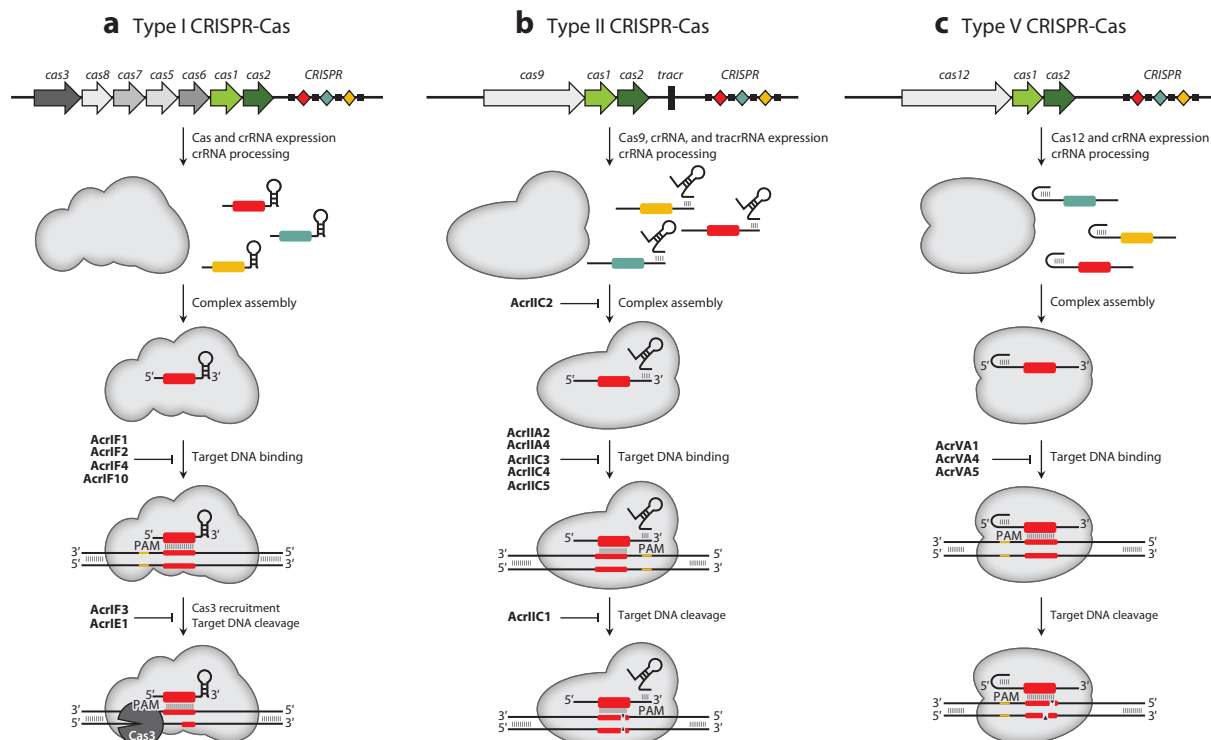
Figure 1

Anti-CRISPR (Acr) proteins inhibit CRISPR-Cas systems at distinct stages. (*a*) A generalized type I CRISPR-Cas locus is depicted. *cas* (CRISPR-associated) genes and the CRISPR array are expressed. The array transcript is subsequently processed into mature crRNA. Cas proteins assemble around the mature crRNA to form the CRISPR-Cas complex, which identifies foreign DNA targets through complementary base pairing with its crRNA and an appropriate PAM sequence. AcrIF1, AcrIF2, AcrIF4, and AcrIF10 prevent the CRISPR-Cas complex from interacting with DNA. Annealing of the crRNA triggers R-loop formation, the recruitment of the Cas3 helicase/nuclease, and the destruction of the DNA target. AcrIF3 and AcrIE1 disable Cas3 to prevent target cleavage. (*b*) A generalized type II CRISPR-Cas locus is depicted. Cas9 and processed crRNA and tracrRNA assemble in a complex that can recognize protospacer adjacent motif (PAM)-flanked sequences with complementarity to its crRNA. AcrIIC2 inhibits crRNA loading into Cas9, preventing proper complex assembly. AcrIIA2, AcrIIA4, AcrIIC3, AcrIIC4, and AcrIIC5 inhibit the complex from recognizing target DNA. Following target recognition, Cas9 creates a double-stranded DNA break target, leading to its destruction. AcrIIC1 inhibits the nuclease activity of Cas9 to prevent target cleavage. (*c*) A generalized type V CRISPR-Cas locus is depicted. Cas12 and the crRNA are expressed, processed, and assembled into a surveillance complex. The complex binds target DNA with a crRNA-complementary, PAM-flanked target. AcrVA1, AcrVA4, and AcrVA5 prevent DNA recognition. Target binding triggers the nuclease activity of Cas12, generating a staggered double-stranded DNA break to destroy the target. Colored arrows represent *cas* genes. In the CRISPR array, black boxes represent repeats, and colored diamonds represent spacers. The PAM is shown in yellow. *cas1* and *cas2* encode proteins involved in CRISPR adaptation.

CRISPR-Cas SYSTEMS

CRISPR loci are composed of two parts: The first is an array comprising variable spacers that are primarily derived from foreign genetic elements separated by a palindrome that is repeated throughout the array; the second is a group of *cas* genes encoding proteins associated with the array (for a review see 8). This array is transcribed, processed, and complexed with Cas proteins (**Figure 1**). The resulting CRISPR-Cas complex contains one CRISPR RNA (crRNA) bound to one or more Cas proteins that can specifically recognize invading foreign genetic elements/nucleic acids through complementarity with the crRNA. The site on the invading DNA that is targeted

by the spacer, known as the protospacer, is usually flanked by a protospacer adjacent motif (PAM), which is generally composed of two to four nucleotides and is specific for a given CRISPR-Cas system. In most cases, PAMs are crucial in allowing CRISPR-Cas systems to distinguish self from nonself and prevent the binding of CRISPR complexes to the CRISPR arrays from where they originated. Once foreign nucleic acids are bound, the CRISPR-Cas complex mediates their cleavage and subsequent destruction (**Figure 1**). A unique power of CRISPR-Cas systems is their ability to adapt to invading MGEs that have not been previously encountered. This is accomplished by excising short sequences from these elements and integrating them into the CRISPR array as new spacers. Thus, immunity to these MGEs is acquired. Despite these commonalities, CRISPR-Cas systems are highly variable—genetically, architecturally, and functionally (9).

The diverse CRISPR-Cas systems have been recently categorized into two classes, six types, and more than thirty subtypes, which differ in their palindromic repeats, spacer lengths, and complement of unique Cas proteins (9). In Class 1 systems, a complex of crRNA and at least three different Cas proteins carry out nucleic acid recognition and cleavage. In Class 2 systems, a single Cas protein bound to crRNA mediates these functions. The Class 2 systems include the CRISPR-Cas9 systems that are widely used for genome-editing applications (for a review see 10). Acrs that block CRISPR-Cas types of both classes have been found.

DISCOVERY OF ANTI-CRISPRs

Initial Discovery of Anti-CRISPRs

Acrs were first discovered as part of an investigation of the effects of prophages in the bacterial species *Pseudomonas aeruginosa* (*Pae*). Prophages are formed when a bacterial virus (also known as a bacteriophage or phage) integrates its complete genome into the host genome. Prophage formation is a frequently occurring feature of phages. Although most genes within prophages are silenced, genes that remain expressed often elicit profound physiological effects (11, 12). Bondy-Denomy et al. (13) constructed a collection of strains that each contained a different prophage in an effort to systematically investigate their phenotypic effects. Using this collection, they discovered three prophages that mediated inhibition of the type I-F CRISPR-Cas system present in the *Pae* strain under study (6). By comparing the genomes of these phages to those of related phages, a region potentially containing anti-CRISPR (*acr*) genes was discovered in eight different phages. Ultimately, genes encoding five completely distinct Acr protein families (AcrIF1–AcrIF5) that inhibit the type I-F system were identified. A subsequent study found that genes located within the same phage *acr* region encoded four additional Acr families (AcrIE1–AcrIE4). These Acrs inhibit the type I-E CRISPR-Cas system encoded by some *Pae* strains (14). Remarkably, among these nine newly discovered families, all proteins were fewer than 140 aa in length and displayed no sequence similarity to other putative or known proteins.

Protein sequence database searches using Acr proteins from the initial nine families as PSI-BLAST (15) queries identified only closely related homologs encoded within the *Pseudomonas* genus, making the identification of additional families of Acrs challenging. This problem was circumvented by the observation that one gene, referred to as anti-CRISPR-associated gene 1 (*aca1*), was conserved at the 3′-end of every *acr* region (**Figure 2a**). The Aca1 protein contains a predicted helix-turn-helix (HTH) DNA-binding domain, suggesting that it might be a regulator of the *acr* operon (see below). By performing PSI-BLAST searches with the Aca1 protein, additional Acrs were discovered by identifying small genes that were encoded upstream of *aca1* homologs within prophages and other types of MGEs. In vivo activity assays performed with some of these candidates revealed two additional families of Acrs that inhibited the type I-F system of *Pae* (AcrIF6 and AcrIF7) (16). Fortuitously, the AcrIF6 family emerged as the first to include
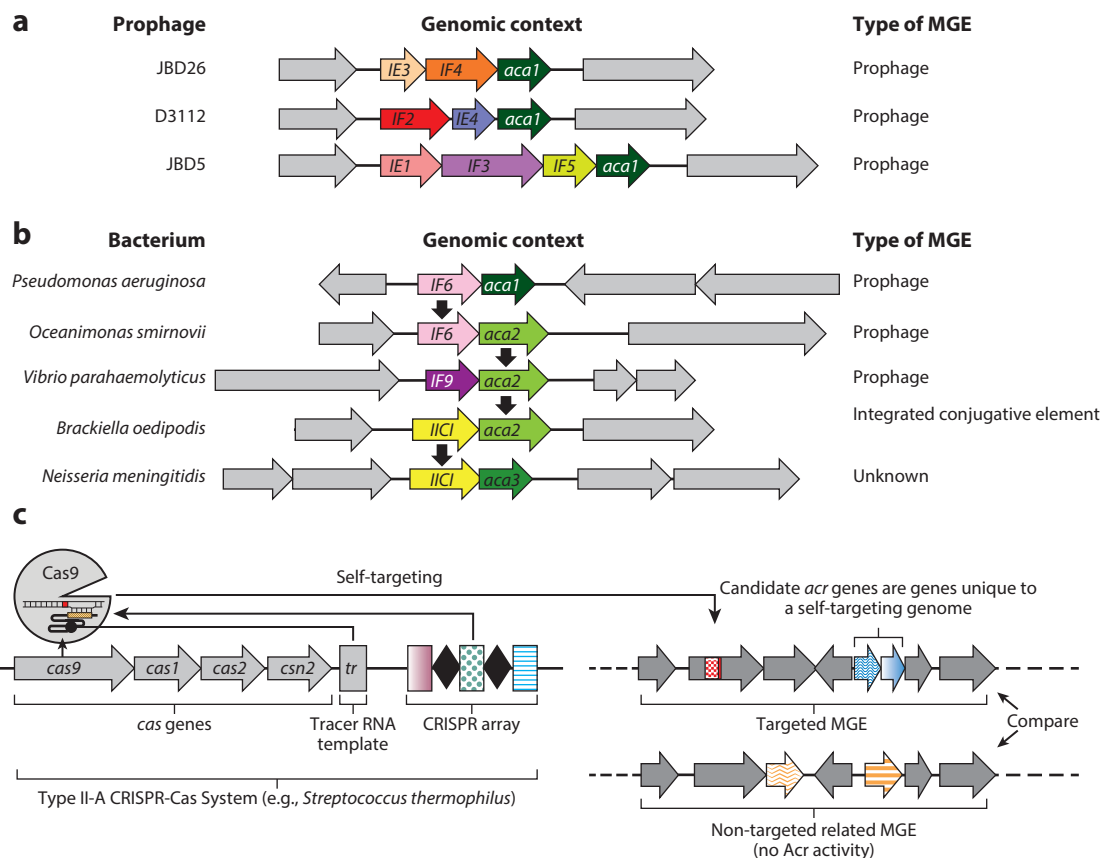
Figure 2

The commonly used approaches for anti-CRISPR (*acr*) gene discovery. (*a*) Representative *acr* regions are shown from the *Pae* phages in which these genes were first identified. Gray arrows depict predicted open reading frames flanking the *acr* regions. Dark green arrows correspond to genes encoding the highly conserved anti-CRISPR-associated gene 1 (*aca1*) predicted to encode a protein with a helix-turn-helix (HTH) motif. The remaining colored arrows represent the *acr* genes. (*b*) The guilt-by-association method is a homology-based computational search approach that uses the proteins encoded by the *acr* and *aca* genes as queries. For example, a search with AcrIF6 (*pink arrow*) of *Pae* identified a homolog in *Oceanimonas smirnovii*. The gene encoding this protein was found adjacent to *aca2*, which is a distinct HTH protein. Searches with Aca2 revealed homologs in the genomes of *Vibrio parahaemolyticus* and *Brackiella oedipodis*, which led to the discovery of *acrIF9* (*purple arrow*) and *acrIIC1* (*yellow arrow*) positioned upstream of *aca2* in these bacteria. Finally, homology-based searches using the protein encoded by *acrIIC1* resulted in the discovery of *aca3* (*dark green arrow*). This back-and-forth approach has facilitated the discovery of many families of Acrs. (*c*) The self-targeting approach is also commonly used for *acr* gene discovery. If a prokaryotic strain has a functional CRISPR-Cas system (e.g., type II-A depicted on the *left*) and contains a spacer (*green dotted box in the CRISPR array*) that matches a protospacer (*red and white checkered box within the dark gray arrow on the right*) located adjacent to a known PAM (*red box within the dark gray arrow on the right*) within a mobile genetic element (MGE), then that organism must also have an *acr* gene—otherwise, its CRISPR-Cas system would degrade its own genome. Black diamonds represent the palindromic repeats in the CRISPR array. Candidate *acr* genes are unique genes (*patterned blue and orange arrows*) present in the targeted MGE (*patterned blue arrows*) and absent from a related MGE that elicits no Acr activity. The dark gray arrows represent genes are conserved in the targeted and non-targeted MGE and are unlikely to be Acrs.

diverse homologs in a wide variety of bacterial species. Unexpectedly, one AcrIF6 homolog was encoded in a prophage region that did not include an *aca1* gene. However, the protein encoded directly downstream of the gene encoding this Acr contained a predicted HTH DNA-binding domain. Homologs of this new putative DNA-binding protein, named Aca2, were found to be

encoded downstream of genes encoding other small proteins that turned out to possess Acr activity (16). This iterative guilt-by-association approach yielded three additional families of Acrs with inhibitory activity against the *Pae* type I-F system (AcrIF8–AcrIF10) (**Figure 2*b***). PSI-BLAST searches with members of these new families detected homologs in bacterial species as broadly distributed phylogenetically as the type I-F system itself, indicating the potential for Acrs to inhibit most of the known I-F systems (16).

Further PSI-BLAST searches using Aca2 as a query identified putative Acrs encoded within MGEs in strains of *Brackiella oedipodis* and *Neisseria meningitidis* (*Nme*) (17). Because these strains encoded no Class 1 systems but encoded type II-C Cas9 systems, the activity of these putative Acrs was tested against the *Nme* CRISPR-Cas9 system. These experiments revealed three families of Acrs (AcrIIC1–AcrIIC3) that inhibited NmeCas9. Excitingly, these Acrs were also shown to inhibit genome editing in cultured human cells (17).

Further Anti-CRISPR Discovery Through Bioinformatics

In addition to the guilt-by-association approach described above, another computational method, the self-targeting approach, has been fruitful in identifying new Acrs (**Figure 2*c***). This approach involves searching for bacterial genomes encoding both a functional CRISPR-Cas system and CRISPR spacers that target sites within the same genome. Such strains would not be expected to survive unless their genomes also encoded Acrs to inhibit their CRISPR-Cas systems. Rauch et al. (18) identified strains of *Listeria monocytogenes* (*Lmo*) bearing self-targeting spacers. Within one of these strains, they also identified a targeted prophage and showed that this prophage conferred Acr activity. Subsequent comparison of this phage genome with a closely related phage genome that did not possess Acr activity facilitated the identification of an *acr* region encoding two proteins (AcrIIA1 and AcrIIA2) that efficiently inhibited the type II-A *Lmo* CRISPR-Cas system. BLAST searches with these Acrs led to additional prophage *acr* regions, and the identification of two more Acr families, AcrIIA3 and AcrIIA4. Importantly, AcrIIA2 and AcrIIA4 were able to inhibit the *Streptococcus pyogenes* (*Spy*) CRISPR-Cas9 system, which is by far the most widely used system for genome-editing applications. This inhibition was demonstrated in both a bacterial assay and human tissue culture cells (18).

In Watters et al.'s subsequent study (19), a systematic search of 150,000 prokaryotic genomes identified more than 9,000 genomes (~6% of the 150,000) with self-targeting spacers. By focusing on self-targeting strains of *Moraxella bovoculi*, which possesses a type V-A CRISPR-Cas12 system, Acrs inhibiting this system (AcrVA1, AcrVA4, and AcrVA5) were discovered. A guilt-by-association approach carried out at the same time also uncovered type V-A Acrs in strains of *Moraxella catarrhalis* (AcrVA1–AcrVA3) (20). These discoveries were notable because type V CRISPR-Cas12 systems are increasingly being used for genome-editing applications (21). In their study, Marino et al. (20) also discovered AcrIC1, an Acr inhibiting the type I-C CRISPR-Cas system.

Functional Screens and Selections to Identify Anti-CRISPRs

In a purely functional approach to Acr identification, Hynes et al. (22) used a collection of hundreds of virulent phages infecting *Streptococcus thermophilus* (*Sth*), a bacterial strain critical in the production of yogurt, to identify phages that did not elicit CRISPR-Cas-based immunity. One of these phages was confirmed to be resistant to CRISPR-Cas attack even when infecting cells producing spacers designed to target the phage genome. By cloning and expressing all of the genes from this phage, one was found, called *acrIIA5*, that inhibited the type II-A systems of the *Sth* and *Spy* CRISPR-Cas9 systems. A subsequent study using the same functional approach

combined with gene neighborhood analysis identified AcrIIA6 (23). A conceptually related approach characterized a CRISPR-Cas-resistant virus infecting the archaeal species *Sulfolobus islandicus*. By isolating viral mutants that became sensitive to CRISPR-Cas, He et al. (24) discovered an Acr that inhibits the type I-D system of this species. This Acr, called AcrID1, is the only type I-D Acr identified so far.

In a recent study, functional selection was also used to isolate genes with Acr activity from human gut and soil metagenomic libraries (25). For the selection, SpyCas9 was coexpressed in *E. coli* with a single-guide RNA (sgRNA) directing the CRISPR-Cas complex to cleave a second plasmid conferring chloramphenicol resistance (Cm^r). Introduction of the Cm^r-conferring plasmid into the strain expressing the SpyCas9:sgRNA complex resulted in very few colonies being resistant to Cm due to cleavage of the plasmid by Cas9. However, expression of AcrIIA2 from the Cm^r-conferring plasmid resulted in thousands of colonies growing in the presence of Cm because the plasmid is protected from SpyCas9-mediated destruction by the Acr. Random fragments of DNA derived from various metagenomic samples were then cloned into the Cm^r-conferring plasmid, allowing for the selection of plasmids encoding proteins that inhibit SpyCas9, based on the bacterium's ability to grow in the presence of Cm. Using this approach, four genes that encoded proteins (AcrIIA7–AcrIIA10) that could inhibit SpyCas9 DNA cleavage activity in vitro were identified. Three of these four putative Acrs also bound robustly to SpyCas9 in vitro (25). The homologs of AcrIIA7 and AcrIIA9 were much more broadly distributed among diverse bacterial phyla compared to other previously identified Acrs. Surprisingly, these homologs were not enriched for occurrence in species encoding CRISPR-Cas systems of the same subtype (in this case, type II-A) implicated at the time of discovery, which has been a consistent feature of other Acr families. AcrIIA8 displayed sequence similarity and predicted structural similarity to a phage structural protein involved in joining the phage head to the phage tail (26, 27). Our own synteny analysis of the closest homolog of AcrIIA8 in the NCBI database (refseq ID WP_009270720; 80% identical to AcrIIA8), which lies in a prophage of *Clostridium butyricum*, indicates that it must function as a head-tail joining protein in this phage. In future studies, it will be of great interest to characterize the Acr activity of homologs of these functionally selected Acrs to determine whether these are families of Acrs or whether the detected Acr activity of the proteins isolated may be adventitious, and not reflect their biologically relevant function.

Nomenclature for Anti-CRISPR Proteins

Bondy-Denomy et al. (7) recently published a standard naming convention for Acrs. Acr familes are named for their type (e.g., IF) and are numbered sequentially as they are discovered, so that, for example, the third Acr family blocking a type I-F system is named AcrIF3. To avoid redundancy in names, investigators submit the names for new Acr families as soon as the paper describing them has been accepted. All Acr names are listed in a spreadsheet (**https://tinyurl.com/anti-CRISPR**), which also lists other useful information about the Acr family, such as publication information and amino acid sequence.

Small-Molecule Inhibitors of CRISPR-Cas Function

Due to the many practical applications for Cas9 inhibitors in genome-editing applications, some investigators have focused on developing small-molecule Cas9 inhibitors, as these would have some advantages over proteins. Maji et al. (28) generated a platform to screen large libraries of small molecules to identify inhibitors of SpyCas9. This high-throughput platform screened small-molecule libraries with a fluorescence-polarization-based assay for DNA binding. One molecule, BRD0539, was identified as a SpyCas9 inhibitor that is stable in human plasma. This molecule

demonstrated dose and temporal control of SpyCas9 in vitro, in both bacterial and mammalian cells. This approach will likely be useful for identifying small-molecule inhibitors for other existing and emerging CRISPR-associated nucleases.

MECHANISMS OF ANTI-CRISPR FUNCTION

The tremendous significance of CRISPR-Cas systems for genome-editing applications has driven a correspondingly large effort directed at understanding the mechanisms by which these systems are inhibited by Acrs. The ability of these small proteins to potently block the activity of large ribonucleoprotein complexes is remarkable, and elucidating the many different means by which this can be achieved provides fascinating new insights into the functioning of CRISPR-Cas systems. Below we describe the best characterized mechanisms by which Acrs inhibit type I, type II, and type V CRISPR-Cas systems. **Figure 3** provides a gallery of all of the solved Acr structures.

## MECHANISMS OF TYPE I ANTI-CRISPR ACTIVITY

Being the first discovered, Acrs inhibiting the *Pae* type I-F system were also the first to be characterized in vitro (29). The 350 kDa DNA recognition complex of the type I-F system, known as the Csy complex, is composed of four Cas proteins arrayed along a single 60-nucleotide crRNA (**Figure 4**). Cas6f, which is the nuclease responsible for processing the CRISPR array transcript into single crRNAs, remains bound to a stem-loop at the 3′-end of the crRNA. Six copies of Cas7f form the backbone of the complex, binding nonspecifically to the spacer region of the crRNA. A complex of Cas8f and Cas5f binds to the 5′-end of the crRNA, known as the handle (30, 31). The Csy complex searches for a canonical PAM site specific for the type I-F system (3′-GG-5′) and, once found, unwinds the DNA duplex to form a stable R-loop where the target strand hybridizes with the crRNA and the nontarget strand is stabilized by the Cas8f subunit. Cryo-electron microscopy (cryo-EM) structural analysis of the DNA-bound Csy complex shows a large conformational change in the complex involving the elongation of the Cas7f backbone and rotation of the Cas8f subunit relative to the non-DNA-bound form (31, 32). The elongation of the Cas7f backbone is required to form stable interactions between the crRNA and the target strand, and the Cas8f rotation is required to expose the R-loop-binding channel for nontarget strand stabilization as well as to expose the docking site for Cas3, the nuclease of type I systems. Cas3 is recruited to the Csy complex and binds to Cas8f following formation of the R-loop (32).

Inhibition of DNA Binding

In vitro studies using purified components showed that AcrIF1, AcrIF2, and AcrIF4 bind directly to the Csy complex (29). AcrIF3 did not bind to the Csy complex, but bound Cas3, thereby preventing the nuclease component of the system from being recruited to the Csy:DNA complex. The binding of either AcrIF1 or AcrIF2 to the Csy complex abrogated its DNA-binding activity. However, these Acrs blocked DNA binding in different ways, with AcrIF1 binding to Cas7f subunits and AcrIF2 binding to the Cas8f subunit. Furthermore, AcrIF1 was found to completely occlude the DNA-binding site of the Csy complex, whereas complementary ssDNA molecules could still bind to the 3′-end of the spacer RNA in the presence of AcrF2, even though this Acr blocked binding of dsDNA and ssDNA complementary to the 5′-end of the spacer. AcrF2 was found to compete with dsDNA for binding of the Csy complex, whereas AcrF1 was still able to bind to the Csy complex presaturated with DNA.

Structures determined by cryo-EM confirmed that AcrIF1 binds to Cas7f, showing two molecules per Csy complex bound at the interfaces formed by the pairs of Cas7f subunits closest
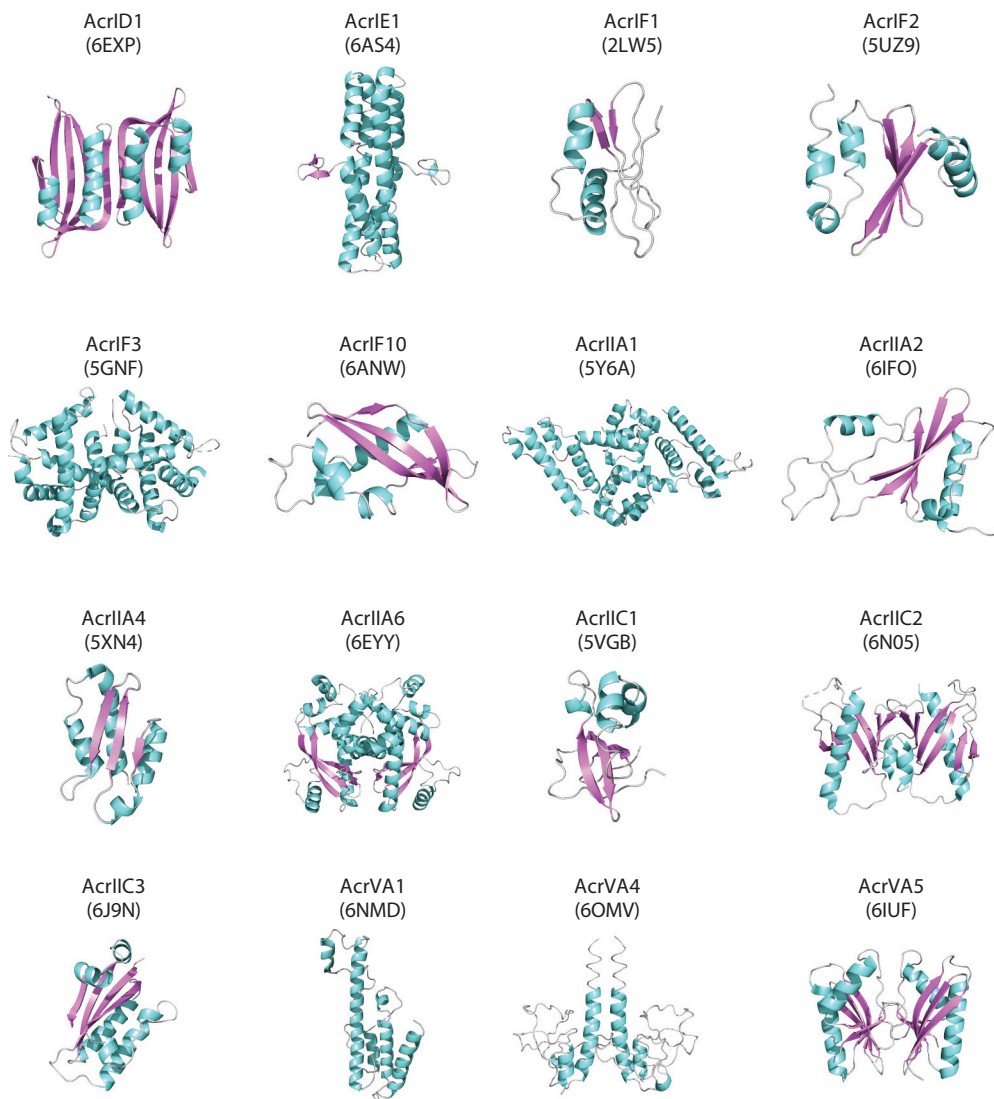
Figure 3

All of the solved structures of anti-CRISPRs (Acrs) are shown. Some of these were solved on their own, and some were solved bound to CRISPR-Cas complexes. No significant structural changes have been observed between the bound and unbound forms of Acrs when both have been solved. The Protein Data Bank identification codes for each structure are shown in parentheses.

to the 5′-end of the crRNA (**Figure 4a**) (30, 31, 33). Binding of AcrIF1 prevents target DNA binding by sterically hindering access to the DNA-binding groove. Prior to the cryo-EM studies, the structure of AcrIF1 alone was determined by nuclear magnetic resonance (34). Structure-guided mutagenesis experiments identified three residues (Y6, Y20, and E31) that are critical for binding to the Csy complex and for inhibiting CRISPR-Cas activity (34). In the cryo-EM structures, these residues were found to be deeply buried in the interface between AcrF1 and Cas7f, interacting with K85, a conserved residue in Cas7f. A K85A substitution markedly weakened the interaction between AcrIF1 and the Csy complex (30).

As was shown biochemically (29), cryo-EM structures revealed a single molecule of AcrIF2 bound to the Cas8f subunit of the Csy complex (**Figure 4***b*). The surface of AcrIF2 is negatively charged, facilitating its interaction with positively charged residues in a lysine-rich vise formed at the interface between Cas8f and the closest Cas7f subunit (Cas7.6f). This region on the Csy complex is crucial for DNA binding (30). AcrIF2 was originally thought to act as a DNA mimic by competing with DNA for binding at the lysine-rich vise (30). However, further cryo-EM analysis revealed that although AcrIF2 binds at the interface between Cas8f and Cas7.6f, it only partially overlaps with the region occupied by the DNA. AcrIF2 most likely inhibits the Csy complex by

(*Caption appears on following page*)
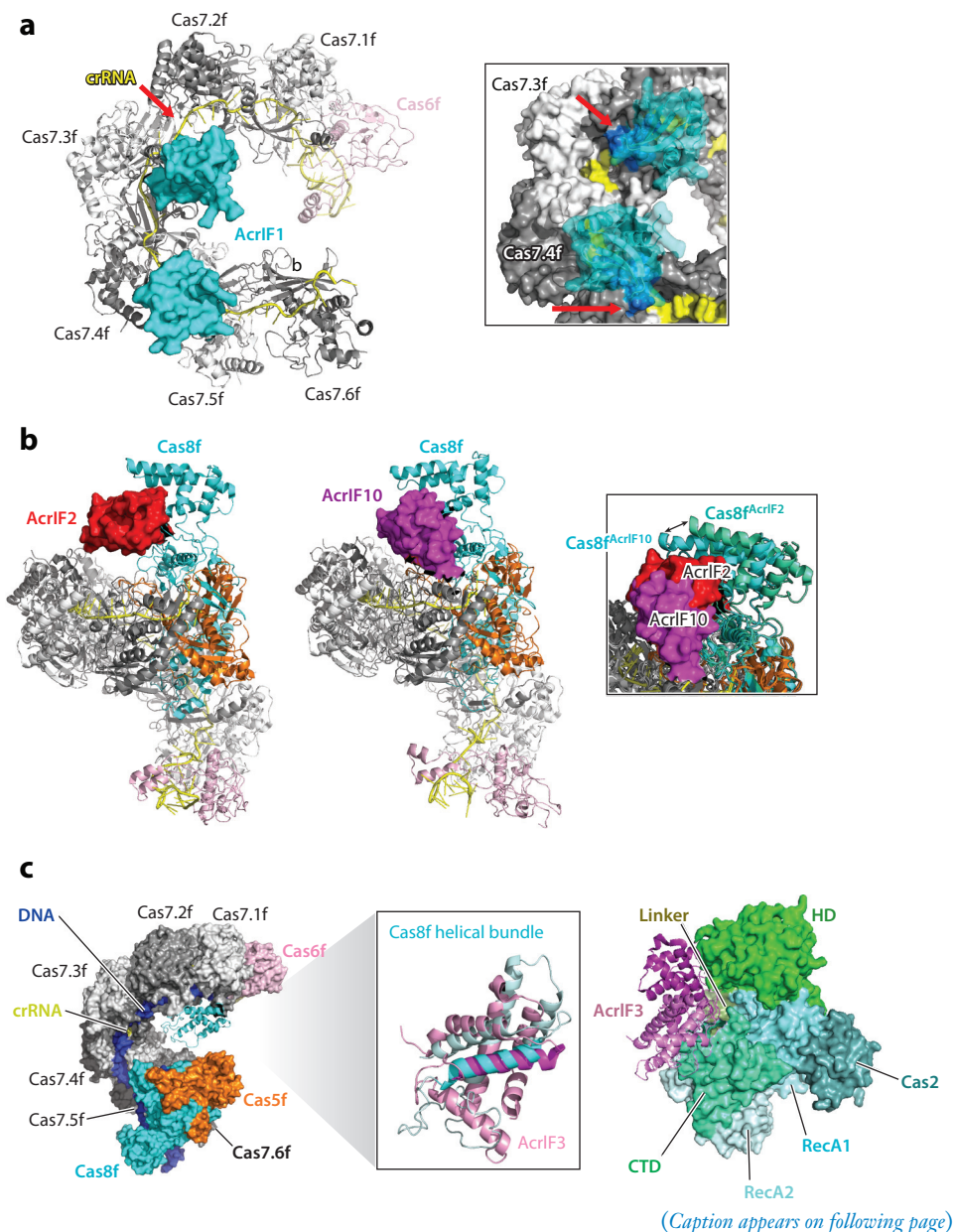
Figure 4 (*Figure appears on preceding page*)

Structures of type I-F anti-CRISPRs (Acrs) bound to the *Pae* Csy complex determined by cryo-EM. (*a*) The cryo-EM structure of AcrIF1 bound to the Csy complex in the presence of a crRNA (*yellow*) is shown. Two copies of AcrIF1 (*cyan*) bind to the Cas7f backbone (*gray*) of the complex. Cas8f and Cas5f are not shown here for simplification. On the right is shown a close-up where residues 8–15 and 33–35 of AcrIF1 (*blue spheres*) reach into the DNA-binding groove to sterically clash and prevent target DNA hybridization. Red arrows indicate these residues. (*b*) The left image shows AcrIF2 (*red*) bound to the Cas8f hook domain (*cyan*) of the Csy complex. The middle image shows the structure of AcrIF10 (*magenta*) bound to the Cas8f hook domain (*cyan*). The right image shows that the two Acrs bind in different locations and cause the hook to move in different directions. (*c*) The left image shows the complete Csy complex. The helical bundle of Cas8f that binds to Cas2–3 is in cyan in the center of the structure. In the middle image, this bundle is overlaid with AcrIF3. The two helices that overlay well and are in bolder colors are both critical for binding to Cas2–3. On the right is the structure of Cas2–3 bound to AcrIF3. Abbreviations: cryo-EM, cryo-electron microscopy; CTD, C-terminal domain; HD, N-terminal histidine-aspartate.

swinging a flexible domain of Cas8f, known as the hook, away from Cas7.6f, resulting in a regional conformational change that is incompatible with DNA binding (31).

The structure of the Csy complex bound to AcrIF10 (35), an Acr which had not been previously characterized in vitro, revealed that it occupies the same site as the DNA duplex and likely acts as a DNA mimic (**Figure 4*b***). The presence of AcrIF10 induces a local conformational change in the Cas8f hook domain, causing it to swing toward Cas7.6f, locking Cas8f in a similar conformation to that seen upon DNA binding. Although AcrIF10 and AcrIF2 both bind to the interface between Cas8f and Cas7.6f, the regions that they occupy overlap minimally, and they cause the Cas8f hook to swing in opposite directions (**Figure 4*b***) (31).

The structure of AcrID1 has also been solved (**Figure 3**), and it has been shown to bind Cas10d, the large subunit of the I-D CRISPR-Cas complex (24). Because Cas10d has nuclease activity and is involved in DNA binding, it cannot be predicted at this time which step of CRISPR-Cas function is blocked by this Acr.

Inhibition of DNA Cleavage

In contrast to the Acrs discussed above, AcrIF3 binds to the Cas2–3 nuclease. The fusion of Cas2 (a protein involved in adaptation) and Cas3 (a nuclease critical for interference) is a unique feature of type I-F and type I-F variant CRISPR-Cas systems (9). The structure of the complex of this Acr with PaeCas2–3 has been solved by X-ray crystallography (36) and cryo-EM (37) (**Figure 4*c***). Cas2–3 consists of an N-terminal histidine-aspartate (HD) type nuclease domain, two tandem RecA-like subdomains (RecA1 and RecA1) that together make up the SF2 helicase domain, a linker region, and a C-terminal domain (CTD). AcrIF3, an all-helical protein, binds as a dimer to Cas2–3, covering the HD domain, linker region, RecA2, and the CTD. This interaction blocks the DNA-binding tunnel formed by RecA2 and the CTD and prevents Cas2–3 from accessing substrate DNA. Furthermore, AcrIF3 masks most of the exposed surface between the CTD and linker region that is required for Cas2–3 binding to the Cas8f subunit of the Csy complex after DNA target binding.

Cryo-EM characterization of the Csy complex bound to a dsDNA target sequence showed that DNA binding is accompanied by a large conformational change in a helical bundle domain of Cas8f, which results in a dramatic repositioning of this region (32). This domain forms the interface for binding to Cas2–3 (**Figure 4*c***). Remarkably, this Cas8f helical domain resembles the structure of AcrIF3 with one central helix displaying five identical residues (**Figure 4*c***). Substituting some of these residues at structurally comparable positions in Cas8f or AcrIF3 abrogated

binding of both proteins to Cas2–3. Thus, AcrIF3 appears to be a structural mimic of the Cas8f domain that recruits Cas2–3 to the DNA-bound Csy complex.

Detailed mechanisms for Acrs inhibiting other type I systems have not been elucidated. However, the mode of action of AcrIE1 (14), which blocks the *Pae* I-E system, has been shown to be similar to AcrIF3. This Acr also binds to the nuclease (Cas3) and likely prevents its recruitment to the DNA-bound I-E CRISPR-Cas complex. The structure of this Acr, determined by crystallography (35), is comprised of a dimer with each subunit containing three α-helices and a small C-terminal β-sheet (**Figure 3**). Apart from being all helical, the AcrIE1 structure does not resemble that of AcrIF3, suggesting that they inhibit Cas3 and Cas2–3, respectively, in different ways.

A notable feature of AcrIF3 and AcrIE1 is that these Acrs do not prevent specific binding of the CRISPR-Cas complex to its DNA target. Thus, these Acrs convert the CRISPR-Cas system into a specific DNA-binding complex that could perform other functions within the cell, such as repressing transcription. The ability of the Csy complex to specifically repress transcription in the presence of AcrIF3 has been demonstrated (6).

MECHANISMS OF TYPE II ANTI-CRISPR ACTIVITY

Type II CRISPR-Cas systems use a single effector protein, denoted Cas9, and are thus designated as Class 2. These systems are by far the most widely studied due to their proven utility in diverse genome-editing applications (10, 38). For this reason, mechanistic studies of Acrs specific for these systems have been pursued with great vigor.

There are three subtypes of the type II system: II-A, II-B, and II-C. These subtypes are distinguished based on CRISPR-Cas locus architecture and Cas9 phylogeny. Cas9 is a multi-domain protein that includes both REC and NUC lobes (**Figure 5a**) (for reviews see 39–42). In its natural context within the cell, Cas9 binds to two separate RNA molecules, the crRNA, which is processed from the CRISPR array and includes the spacer region, and a constant tracrRNA, which sits adjacent to the CRISPR array in the CRISPR-Cas locus and is required for crRNA processing. In most Cas9-based applications the crRNA and tracrRNA are joined into one molecule called the sgRNA. The REC lobe is primarily responsible for recognition of sgRNA with an arginine-rich bridge helix (BH) domain playing a particularly important role. The NUC lobe contains the two domains responsible for Cas9 cleavage of target DNA: the HNH domain that cleaves the crRNA-complementary DNA strand and the three-part RuvC domain (RuvC-I, RuvC-II, and RuvC-III) that cleaves the noncomplementary DNA strand. At the C-terminal end of Cas9, also within the nuclease (NUC) lobe, are the wedge domain (WED) and PAM-interacting domain (PID), which mediate initial DNA-binding and PAM recognition. Cas9 functions by first binding to sgRNA, which triggers a large conformational change, resulting in formation of the DNA surveillance complex. This complex scans for its cognate PAM sequence and, upon detection, surveys adjacent DNA for potential target sequences. Once Cas9 identifies target DNA with the correct PAM, an R-loop forms and the HNH domain is activated for cleavage of the target strand. Structural change of the HNH domain indirectly induces activation of the RuvC domain, which then cleaves the nontarget strand.

Inhibition of DNA Binding

AcrIIA4 was the first type II Acr to be characterized in detail. Highlighting the intense interest in this type of Acr, four papers describing the AcrIIA4 structure were published in less than a year, with the first emerging only four months after its discovery (43–46). Structures of AcrIIA4 in complex with sgRNA-bound SpyCas9 show that this highly acidic protein binds the PID of
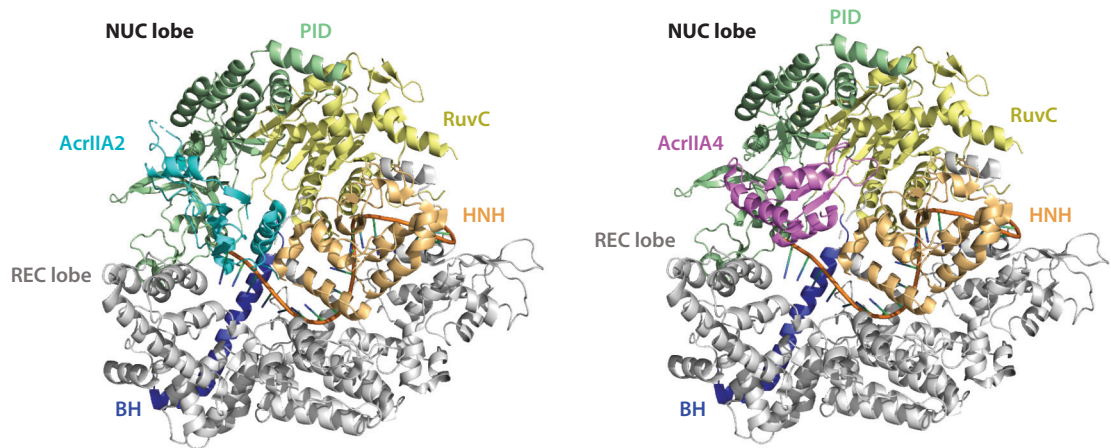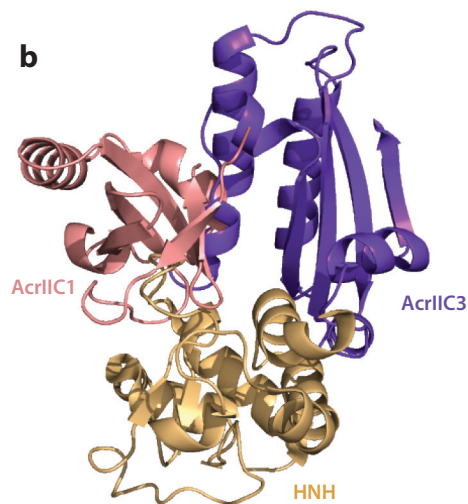
Figure 5

Structures of Cas9 bound to anti-CRISPRs (Acrs). (*a*) The structure on the left shows SpyCas9 bound to AcrIIA2 (*cyan*) primarily interacting with the PAM-interacting domain (PID) of Cas9 (*pale green*) as well as the HNH (*light orange*) and REC domains (*gray*). The structure on the right shows SpyCas9 bound to AcrIIA4 (*violet*) primarily interacting with the PID (*pale green*) as well as the RuvC domain (*pale yellow*). Both Acrs bind in the same region, which is also where DNA binds. (*b*) AcrIIC1 (*pink*) and AcrIIC3 (*purple*) interact with different surfaces of the HNH domain (*light orange*) from NmeCas9. Additional abbreviations: BH, bridge helix; NUC, nuclease.

Cas9, mimicking the PAM region of the dsDNA target and preventing DNA binding (**Figure 5a**) (43, 45, 46). AcrIIA4 also interacts with residues within the RuvC active site, so that nuclease activity would likely also be abrogated if DNA were able to bind. The effectiveness of AcrIIA4 as an inhibitor is emphasized by its strong binding affinity for Cas9:sgRNA (K_d = 4 nM), which is 15-fold higher than the Cas9:sgRNA affinity for target DNA (43). AcrIIA4 binds at least 1,000-fold more weakly to Cas9 alone than to Cas9:sgRNA (44), confirming that the conformational changes occurring upon sgRNA binding are required to form the binding site for AcrIIA4, as was deduced from structural comparisons (43).

The mechanism of AcrIIA2 has also been characterized in detail both structurally and biochemically (47, 48). Although AcrIIA2 is entirely unrelated to AcrIIA4 in both sequence and structure, it interacts with the PID of Cas9 through almost identical local contacts as AcrIIA4 (**Figure 5a**). These Acrs compete with DNA for binding to Cas9 and also compete with each other. They present a striking example of convergent evolution where different proteins have been selected to bind to the same sites in a similar manner. While displaying overlap with AcrIIA4 in binding the PID, AcrIIA2 also interacts with the HNH and REC2 domains, which are not contacted by AcrIIA4. A distinguishing feature of AcrIIA2 is its temperature sensitivity, displaying a substantially lower ability to inhibit in vitro Cas9 activity at 37°C compared to 22°C (47). A homolog of AcrIIA2, designated AcrIIA2b and bearing approximately 35% sequence identity to AcrIIA2, is not temperature sensitive and is a much more potent inhibitor of Cas9 than AcrIIA2. Structural characterization of this homolog revealed that two key aromatic residues found only in AcrIIA2b lie close to PAM-interacting residues of Cas9 and are responsible for the increased activity of this Acr.

Type II-C inhibitors AcrIIC4 and AcrIIC5 have been shown in vitro and in vivo to block DNA-binding activity, but not the binding of Cas9 to its corresponding sgRNA (49). Further mechanistic details about these Acrs have yet to be published.

Inhibition of DNA Cleavage

AcrIIC1, originally identified as an inhibitor of the type II-C NmeCas9, was found to inhibit the diverse type II-C Cas9 orthologs from *Campylobacter jejuni* (*Cje*) and *Geobacillus stearothermophilus* (*Geo*), which are 42% and 36% identical to NmeCas9, respectively. Protein interaction studies with domains of GeoCas9 showed that AcrIIC1 binds to the HNH domain. A crystal structure of this Acr bound to the HNH domain of NmeCas9 revealed that it binds to the active site surface of the HNH domain by interacting with several highly conserved residues required for catalysis (**Figure 5b**). Consistent with its binding to a domain responsible for nuclease activity, Cas9:sgRNA bound by AcrIIC1 is still able to bind to target DNA with normal affinity but is unable to cleave the DNA. In this way, AcrIIC1 creates a catalytically dead Cas9 (dCas9) that still binds its DNA target. The ability of AcrIIC1 to inhibit diverse Cas9 orthologs is explicable, as the HNH domain is one of the most conserved regions of Cas9.

Inhibition Through Dimer Formation

AcrIIC3 inhibits DNA binding by NmeCas9 and induces it to dimerize, forming a 2:2 complex of Cas9 and the Acr (50, 51). AcrIIC3 has been shown to bind the HNH domain of Cas9 and structures of this complex have been solved (51, 52). Strikingly, AcrIIC3 binds on the opposite side of the HNH domain from AcrIIC1 (**Figure 5b**). The structure of the AcrIIC3:NmeCas9 2:2 complex shows that AcrIIC3 holds the HNH domain in an inactive state far from its cleavage target (53). In addition, Cas9 dimerization by AcrIIC3 is mediated through its ability to bind a portion of the REC domain in one Cas9 and the HNH domain of the other, thus forming a bridge.

The ability of AcrIIC3 to inhibit only NmeCas9, as compared to the broad specificity of AcrIIC1, is likely explained by the much higher sequence variability observed on the surface bound by AcrIIC3. Supporting this conclusion, substitution of amino acids in the NmeCas9 HNH domain with those found in Cas9 orthologs that are not inhibited by AcrIIC3 resulted in large decreases in binding affinity for this Acr (52). Further interaction studies have indicated that AcrIIC3 also binds the REC lobe. This lobe, which is highly variable in sequence among Cas9 homologs, is required for AcrIIC3-mediated dimerization (51). Because the Cas9 surface bound by AcrIIC3 is not critical for function, it is probably the dimerization mediated by AcrIIC3 that

reduces the DNA-binding affinity of NmeCas9 (50). Important functional surfaces may be buried in the Cas9 dimer interface and/or critical conformational changes may be impeded.

Inhibition of Single-Guide RNA Binding

Protein interaction studies with truncated forms of Cas9 showed that AcrIIC2 binds to the Arg-rich BH in the REC lobe of NmeCas9, which is involved in sgRNA interaction (51, 54). Consistent with this binding site, an AcrIIC2:Cas9 complex is blocked from interaction with sgRNA. Additionally, AcrIIC2 binds weakly to Cas9 once the latter is bound to sgRNA. The structure of AcrIIC2 is dimeric with a large negatively charged surface that serves as the interface for binding the positively charged BH. Co-crystal structures of AcrIIC2 bound to the BH identified key interacting negative residues on the Acr and key positive residues on Cas9 that were verified by mutagenesis (51, 54). Structural superposition of the BH with that of the NmeCas9:sgRNA binary complex shows that the AcrIIC2 dimer sterically clashes with stem-loops 1 and 2, and slightly overlaps the seed region of the sgRNA, altogether blocking the binding sites of the sgRNA to the BH. When AcrIIC2 is coexpressed with Cas9 in *E. coli*, the total accumulation of Cas9 is markedly decreased, likely because Cas9 is locked in its apo-form lacking sgRNA, which is more susceptible to digestion by intracellular proteases. Thus, AcrIIC2 may hamper Cas9 activity in the cell by blocking sgRNA binding and by decreasing the steady-state levels of Cas9.

## MECHANISMS OF TYPE V ANTI-CRISPR ACTIVITY

Belonging to Class 2, type V CRISPR-Cas systems target dsDNA using the effector protein, Cas12. Similar to Cas9, Cas12 forms a structure comprised of two lobes called REC and NUC (for reviews see 39, 41). Cas9 and Cas12 both utilize a RuvC-like endonuclease domain to mediate DNA cleavage. However, unlike Cas9, Cas12 generates staggered cuts and lacks a second nuclease (e.g., HNH) domain. Outside of the RuvC domain, Cas9 and Cas12 bear little or no similarity either in sequence or structure, even though analogous nomenclature is used in naming the domains of these two enzymes (e.g., WED, PAM-interacting, and BH).

Inhibition of DNA Binding with Enzymatic Activity

AcrVA1 is a broad-spectrum inhibitor of type V systems with in vitro and in vivo activities against four diverse Cas12a orthologs, which range in pairwise percent identities between 30% and 40% (19, 20). The mechanism of Cas12a inhibition by AcrVA1 has been explored in biochemical and structural detail (55, 56). AcrVA1 binds Cas12a:crRNA in the cleft between the REC and NUC lobes, interacting mostly with domains involved in PAM interaction, thereby acting as a DNA mimic. Strikingly, AcrVA1 also cleaves the crRNA and permanently prevents DNA binding. AcrVA1-mediated crRNA cleavage is specific to Cas12a-bound crRNA and is independent of spacer sequence or length. Furthermore, crRNA cleavage occurs in the presence of a catalytically dead version of Cas12a, implying that AcrVA1 is not altering the nuclease behavior of Cas12a, but rather has nuclease activity itself. Consistent with this hypothesis, AcrVA1 can perform multiple rounds of crRNA cleavage in the presence of a Cas12a:crRNA complex, making AcrVA1 the first Acr protein demonstrated to have an enzymatic mechanism (55).

AcrVA5 also inhibits the DNA-binding activity of Cas12a, but functions against a more limited set of orthologs as compared to AcrVA1. Surprisingly, this Acr does not form a stable complex with the *Moraxella bovoculi* (*Mb*) Cas12a ortholog that it inhibits (57). This observation led to a search for covalent modifications of *Mb*Cas12a mediated by AcrVA5, and it was discovered that this Acr mediates acetylation of K635. This residue is crucial for PAM interaction, so that
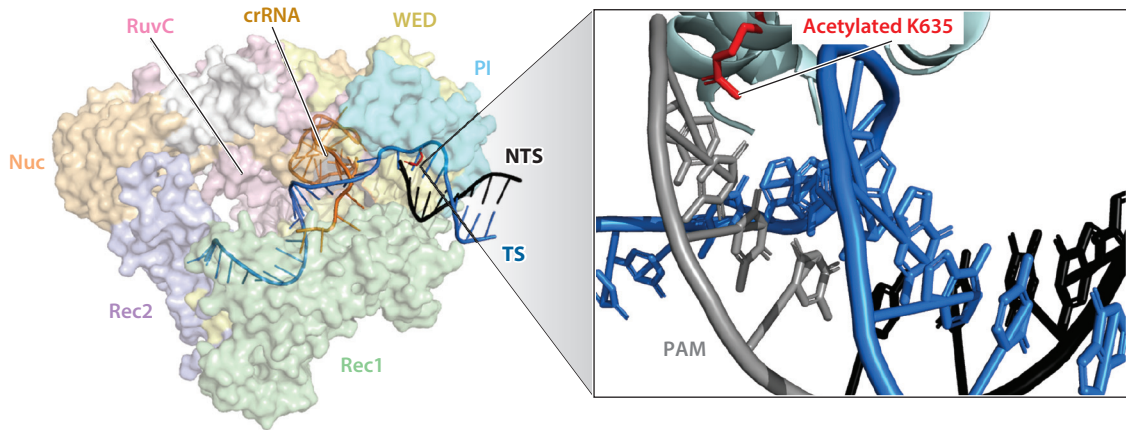
Figure 6

AcrVA5 acetylates K635 and prevents MbCas12a from recognizing the PAM. On the left is a superposition of PAM-bound LbCas12a and MbCas12a acetylated at K635. The close-up on the right shows that the acetylated K635 (*red*) sits within the PID and can sterically block recognition of the PAM. Thus, MbCas12a is unable to interact with target DNA when K635 is methylated. Abbreviations: NTS, nontarget DNA strand; PAM, protospacer adjacent motif; PID, PAM-interacting domain; TS, target DNA strand; WED, wedge domain.

modification at this position completely abolishes DNA binding (**Figure 6**). An *Mb*Cas12a with an Arg residue substituted position 635 is resistant to the inhibitory activity of AcrVA5, as are Cas12a orthologs that naturally possess Arg at this position. Determining the crystal structure of AcrVA5 revealed that it is similar to other acetyltransferases in structure and it is bound to acetyl-CoA (57). Acetylation represents a unique mechanism of Acr function. However, the means by which AcrVA5 recognizes specific Cas12a homologs before acetylating them remains to be discovered.

Inhibition of DNA Binding Accompanied by Cas12a Dimerization

AcrVA4 induces dimerization of Cas12a and blocks its DNA-binding activity (55, 56, 58). Analysis by cryo-EM shows that AcrVA4 binds primarily to portions of the REC domain that are involved in crRNA binding and pre-crRNA processing, which is also carried out by Cas12a (56, 58). AcrVA4 appears to mimic pre-crRNA (58). The binding of AcrVA4 is far from the DNA-binding site, but it is able to allosterically block the conformational changes that are required for Cas12a to bind target DNA. AcrVA4 is a dimer, and structural data show that Cas12a dimerization mediated by AcrVA4 occurs wholly through interactions of the N-terminal domain of AcrVA4. A version of AcrVA4 consisting of only its CTD, which accounts for all of the binding interactions with Cas12a, was able to efficiently inhibit Cas12a activity without inducing dimerization (58). These data show that the formation of Cas12a dimers is not required for the inhibitory activity of AcrVA4.

SUMMARY OF ANTI-CRISPR MECHANISMS

Over the past four years, studies describing detailed biochemical and/or structural characterization of 16 different Acrs have been published. Reflecting the excitement and competition in the CRISPR-Cas field, more than half of these structures have been solved at least twice, and some up to four times. On the bright side, these repeated studies on the same proteins have consistently been in agreement, so we can be confident in our conclusions about Acr mechanisms, which are

summarized in **Figure 1**. Remarkably, among the structures of 16 different Acrs (**Figure 3**), none show any similarity to any others, reflecting a unique evolutionary origin for each Acr family. Despite this structural diversity, there is some mechanistic overlap. Most common is the binding of Acrs at or close to the PAM-interaction region, a property shared by AcrIF2, AcrIF10, AcrIIA2, AcrIIA4, and AcrVA1. These Acrs are all acidic and, to varying extents, mimic DNA. DNA mimicry is a common theme among many inhibitors of DNA-binding proteins including phage-encoded inhibitors of restriction enzymes (59, 60). The second most common activity for Acrs to block is DNA cleavage with AcrIF3, AcrIE1, and AcrIIC1 possessing this activity. Besides AcrIIC2, no Acr blocks biogenesis of the CRISPR-Cas complex. It appears that, in general, Acrs have the most utility if they can directly block the activity of already formed CRISPR-Cas complexes, which would usually be present when foreign DNA is introduced into a cell. The recent findings of Acrs with enzymatic activity is very exciting and we expect that more such examples will arise.

IN VIVO FUNCTION OF ANTI-CRISPR AND ANTI-CRISPR–ASSOCIATED PROTEINS

As described above, Aca proteins are frequently encoded at the 3′-end of *acr* gene regions and are highly conserved in MGEs, including prophages. As such, they have served as a key signpost for new *acr* gene identification. There are currently seven families of Aca proteins (20), whose only commonality is an N-terminal HTH DNA-binding domain. Interestingly, the structures of AcrIIA1 and AcrIIA6 (**Figure 3**) also contain HTH domains, indicating that these Acrs may play a dual role as Acr and Aca proteins (18, 23, 61). Consistent with this idea, AcrIIA1 is extremely widespread and is generally encoded at the 3′-end of *acr* regions. Until recently, the function of Aca proteins was unknown, although it seemed likely that they were involved in regulating *acr* genes, as they appeared to be DNA-binding proteins. With respect to regulation of *acr* genes, it was also not known how Acr proteins accumulate quickly enough after phage DNA entry into the cell to protect against CRISPR-Cas complexes that are already present and poised to destroy the phage genome.

Recently, a study elucidated the function of an Aca protein, denoted Aca1, from *Pae* phage JBD30—one of the phages in which Acr proteins were first discovered (62). This study also measured the expression of *acr* genes early in the infection process. It was shown that *acr* genes are quickly expressed to very high levels at the onset of phage infection. This expression, driven by a powerful promoter located immediately upstream of the *acr* genes, is critical for Acr deployment during infection, as mutant phages lacking this promoter fail to replicate in the presence of CRISPR-Cas. Unexpectedly, Aca proteins were shown to be repressors of *acr* transcription, and, in the case of JBD30, this repression is essential for phage survival. In the absence of Aca activity, uncontrolled expression from the *acr* promoter dysregulates the expression of essential genes downstream of the *acr* operon, causing loss of phage viability. This critical function of Aca proteins likely explains their high conservation in *acr* operons. In addition, the positioning of *aca* genes within *acr* operons has likely facilitated the spread of these operons by allowing them to insert into diverse genomic locations without disrupting the surrounding genetic circuitry.

Also related to the question of how Acr proteins are able to accumulate quickly enough after phage DNA entry, two recent studies showed that initial infections by phages producing Acr proteins are unable to completely inactivate CRISPR-Cas systems, and that these phages are inhibited by CRISPR-Cas (63, 64). Although these infections fail, they nonetheless produce Acr protein that progressively accumulates with each unsuccessful infection. Once a critical concentration of Acr protein is reached, the host exists in an immunosuppressed state that is sensitive to subsequent

infection. This cooperative behavior of Acr-producing phages was demonstrated to be a major determinant of replicative success in the presence of CRISPR-Cas; without a sufficient amount Acr protein donated by sacrificial phages, the infection dies out (63, 64). The density of phages needed to mount a successful defense against CRISPR-Cas largely depends on the strength of the Acr protein, with weaker Acr proteins requiring higher phage densities, and on the potency of the CRISPR-Cas system. Although not explored, the number of *acr* genes targeting the same CRISPR-Cas system likely affects the phage population densities needed to inactivate CRISPR-Cas. Many *acr* loci encode multiple distinct *acr* genes targeting the same CRISPR-Cas subtype. Inhibiting the same system in different ways would likely lower the phage densities necessary to neutralize CRISPR-Cas or perhaps even circumvent the need for cooperation to mount a successful infection. Multiple-turnover enzymatic Acrs could also have a similar effect. Although cooperation provides one means by which phages can outpace CRISPR-Cas, it is possible that other strategies exist. Additionally, as phages are thought to exist in heterogeneous populations, it remains to be determined if other members of the community can exploit the immunosuppressed host generated by the Acr-producing phages.

ANTI-CRISPR EVOLUTION AND EVOLUTIONARY IMPACT

To date, more than 40 families of Acr proteins have been discovered. There is little similarity between the various families aside from their small size (typically between 50 to 150 aa) and a stereotypic association with *aca* genes, making it difficult to trace their evolutionary origin(s). The paucity of structural or sequence similarity to known proteins suggests that Acrs may be the product of de novo evolution. An intriguing feature of Acrs is that some families have homologs that are very diverse and appear in widespread species with respect to phylogeny, whereas other families have few homologs that may occur in only one species. Families with limited distribution may have evolved more recently and not had time to spread by horizontal gene transfer, or these families may have narrow specificity, and can only inhibit the CRISPR-Cas system in one species. Thus, there would be no adaptive drive for their retention in other species.

CRISPR-Cas has been shown, in most cases, to drive phages to extinction when host populations target phages with multiple and diverse spacer sequences. However, under similar conditions, a single *acr* gene is sufficient to overcome CRISPR-Cas (65), thereby imposing a strong selective pressure on CRISPR-Cas to mutate and diversify to bypass Acr inhibition. Therefore, it would be expected that key residues involved in Acr interaction would vary among Cas proteins. Indeed, the critical residue of MbCas12a targeted by AcrVA5 is not universally conserved, allowing some MbCas12a homologs to escape from AcrVA5-mediated inhibition (57). In *Nme*, distinct orthologs of Cas9 with different Acr susceptibilities have been identified (66). However, many Acrs target essential residues or block multiple functions of CRISPR-Cas (30, 36, 37, 50), limiting the possibility of mutational escape. This may act as a driving force for the diversification of CRISPR-Cas systems. We expect that, in general, broad specificity Acrs, such as AcrIIC1, would target highly conserved residues in Cas proteins. Substitutions of these residues that might allow for evasion of Acr inhibition would likely also cause loss of CRISPR-Cas function. Thus, it is more difficult for systems to arise that are not inhibited by these Acrs. Although it remains unknown how Acrs may be shaping CRISPR-Cas diversity, these examples suggest that Acrs likely have a profound influence. Additionally, limited mutational escape has the potential to result in the accumulation of multiple CRISPR-Cas systems in a single genome. In a survey, 6% of bacteria and 14% of archaea were found to carry multiple types and subtypes of CRISPR-Cas systems (3).

Because many subtypes of CRISPR-Cas systems are composed of distinct Cas proteins, it might be expected that each individual Acr would inhibit only a single CRISPR-Cas system. In most cases

this is true, but there are exceptions. For example, the AcrIF6 homolog of *Pae* strongly inhibits both the I-E and I-F systems of *Pae* (16), whereas other AcrIF6 orthologs, encoded in diverse Gammaproteobacteria MGEs, lack the cross-subtype dual specificity of AcrIF6$_{Pae}$. Amino acid substitutions were found that abolished only one of this Acr's activities, suggesting that it possesses two separate functional interfaces (16). Additional examples include one AcrVA3 homolog, which inhibits both I-C and V-A systems (20), and AcrIIA5, which inhibits both II-A and II-C type systems (23, 67). These cases present intriguing targets for future mechanistic studies.

In light of the impact of Acrs on CRISPR-Cas systems, it may be expected that new elements participating in the arms race between these systems will be discovered. For example, some strains may possess so called anti-anti-CRISPR mechanisms, as this type of back-and-forth escalation phenomenon has been well documented in the evolution of restriction-modification systems (68). Taking advantage of direct Acr/CRISPR-Cas interactions, bacteria could upregulate intracellular concentrations of Cas protein to titrate away Acrs thereby inactivating their actions. In *Pae*, overexpression of target Cas protein was shown to inhibit phages relying on Acr for infection (29), providing proof of concept for this mechanism. Bacteria could also repress Acr expression using Aca-like proteins. Heterologous expression of Aca1 from a plasmid has been shown to inhibit Acr-dependent phages (62). Finally, bacteria could possess dedicated anti-anti-CRISPRs that interact directly with Acr proteins. These types of inhibitors could function by preventing Acrs from binding their Cas protein target or by mediating their degradation. Further exploration of these potential anti-anti-CRISPR mechanisms will contribute to our understanding of the coevolutionary dynamics between bacteria and phages as well as the evolution of CRISPR-Cas systems.

APPLICATIONS OF ANTI-CRISPR PROTEINS

The rapidly expanding palette of CRISPR-Cas technologies has led to a corresponding motivation to develop tools to control and modulate their activities. Acr proteins targeting type II (Cas9) and type V (Cas12a) effectors have drawn particular interest as they may provide temporal, spatial, or conditional control over established genome-editing systems. One major application of Acr proteins is their use as off-switches for genome editing. Minimizing, if not abolishing, undesired off-target activity is important for CRISPR-Cas9 technology, especially for therapeutic use. Although extensive efforts have led to Cas9 variants with enhanced specificity (69), excessive or prolonged Cas9 activity may increase the likelihood of off-target editing or cytotoxicity, necessitating a means to shut down Cas9 upon achieving a desired outcome. In combination with engineered Cas9 variants and means to regulate Cas9 expression, Acr proteins can act as an additional safeguard to reduce potential adverse effects of Cas9. For instance, timed delivery of AcrIIA4 following SpyCas9-sgRNA editing of a desired target reduces the extent of off-target editing in cells. Limiting the window of SpyCas9 activity likely reduces off-target effects due to differential kinetics of Cas9 on- versus off-target editing (45). Furthermore, as multiplexed CRISPR-Cas gene therapies are developed in an effort to offer treatments for previously untreatable, complex, genetic diseases (70), Acrs will become even more important for mitigating the potentially exponential increase in off-target effects as the number of crRNAs rises.

CRISPR technology has also been applied to the development of gene drives, which are genetic elements that force super-Mendelian inheritance to disseminate desired traits in a population. A prominent example is the ongoing development of female-sterility-inducing gene drives in mosquitoes to eradicate vector-borne diseases such as malaria (71, 72). A means of effective control over the spread of a gene drive after its initial release is highly desirable. Acr proteins could be deployed to put a brake on the propagation of a CRISPR-based gene drive after the parental

driver organisms are released into the relevant ecosystem. As a proof of concept, temporal control of AcrIIA2 and AcrIIA4 using an inducible promoter has been demonstrated to halt or titrate the efficiency of a SpyCas9-based gene drive in yeasts (73).

Nuclease-inactive dCas9 can be used to tether or recruit various effector proteins to genomic sites of interest. For example, chromatin visualization and targeted gene regulation can be achieved via fusion of fluorescent proteins (FPs) and transcriptional activators or repressors to dCas9, respectively. Technologies based on dCas9 not only allow genome manipulation, but also alteration of the epigenome via fusion of DNA demethylation enzymes (e.g., TET) or histone-modifying effectors (e.g., LSD1 or p300) (74). Acrs that limit DNA binding may also be used to regulate the activities of these functional domains. For example, type II Acr proteins were used to control chromosome labeling by dCas9-FP fusions during live-cell imaging (17), as well as demethylation by dCas9-Tet1 fusions in induced pluripotent stem cells (75). Moreover, Acr proteins have enabled programmable and dynamic gene regulation in CRISPR-based synthetic circuits by regulating CRISPRi (CRISPR interference) and CRISPRa (CRISPR activation) (76).

Acr proteins can often tolerate fusion to epitope tags and FPs without compromising their inhibitory potency (73, 77). This offers opportunities to engineer Acr proteins through domain fusions. For instance, an AcrIIA4 hybrid with a light-inducible LOV2 domain has been shown to control SpyCas9- and dSpyCas9-mediated genome and epigenome editing in optogenetics (77). Posttranslational control of Acr proteins was achieved by fusing an inducible destabilization domain that degrades the protein in the absence of an external ligand known as Shield1 (76). Furthermore, posttranscriptional regulation of Acrs by microRNAs that are expressed in certain cell types enables cell-type-specific inhibition of Cas9 activities (78, 79). This strategy was validated for enforcing the tissue specificity of genome editing not only in cultured cells but also in adult mice (80), demonstrating that Acrs can function in mammalian tissues in vivo. Acr proteins also have applications in the development adenoviral vectors for Cas9 delivery in mammalian cells. Recently, a helper-dependent adenovirus (HDAd) vector for transient Cas9 expression in target cells was created (81). By design, these HDAds encode SpyCas9 and a guide that directs the cleavage of the vectors' own genome after transduction of target cells, thereby allowing transient SpyCas9 expression and function. However, self-cleavage during viral production also occurs, leading to genomic rearrangements that make virus production impossible. AcrIIA2 and AcrIIA4 were used to inhibit SpyCas9 from initiating vector self-cleavage during the viral production, thus greatly improving yield (81). A final potential use of Acr proteins is in development of phage therapies as an alternative to antibiotics to treat bacterial infections (82). Phage therapies, however, may be compromised in pathogenic hosts with active CRISPR-Cas systems such as *Pae* (83) and *Nme* (84). Because Acr proteins have been found in these and other pathogens, *acr* genes could be included in the engineering of therapeutic bacteriophages that circumvent multidrug resistance in pathogenic bacteria.

FUTURE PERSPECTIVES

Starting from the first paper in January 2013, there are now (as of March 2020) 129 papers in PubMed mentioning Acr. Remarkably, 97 of these have been published since January 2018, emphasizing the accelerating interest in this field. Despite the rapid accumulation in knowledge about Acrs, we are surely seeing only the tip of the iceberg so far. Given the rapidity with which Acrs that inhibit many different CRISPR-Cas systems have been discovered, we anticipate that many more Acr families will be identified in the near future and that Acrs will eventually be found to inhibit every type of system. With the widespread occurrence of Acrs, it will become crucial to address the question of whether CRISPR-Cas systems are frequently participating in functions outside of

phage defense and whether Acrs participate in these functions by modulating CRISPR-Cas activity rather than complete inhibition. There are already several examples of CRISPR-Cas systems that fulfill noncanonical roles in gene regulation and virulence (85–88). Another area of future interest will be in elucidating more Acr mechanisms. These mechanisms have already proven to be remarkably diverse, and continued focus in this area will provide many more new insights into CRISPR-Cas function and likely many surprises. Finally, CRISPR-Cas systems represent only one of many bacterial antiphage defense mechanisms, and the number of these systems known has greatly expanded in past four years (1, 89–91). Although Acrs have been rapidly discovered and characterized due to the excitement surrounding CRISPR-Cas systems, no inhibitors have yet been discovered for these fascinating new antiphage systems. The discovery approaches applied to Acr systems should be applicable for finding new so-called anti-anti-phage systems, and we expect that exciting new fields of study will emerge from these efforts.

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