REVIEW ARTICLE

Meet the Anti-CRISPRs: Widespread Protein Inhibitors of CRISPR-Cas Systems

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Abstract

The constant selective pressure exerted by phages, the viruses that infect bacteria, has led to the evolution of a wide range of anti-phage defenses. One of these defense mechanisms, CRISPR-Cas, provides an adaptive immune system to battle phage infection and inhibit horizontal gene transfer by plasmids, transposons, and other mobile genetic elements. Although CRISPR-Cas systems are widespread in bacteria and archaea, they appear to have minimal long-term evolutionary effects with respect to limiting horizontal gene transfer. One factor that may contribute to this may be the presence of potent inhibitors of CRISPR-Cas systems, known as anti-CRISPR proteins. Forty unique families of anti-CRISPR proteins have been described to date. These inhibitors, which are active against both Class 1 and 2 CRISPR-Cas systems, have a wide range of mechanisms of activity. Studies of these proteins have provided important insight into the evolutionary arms race between bacteria and phages, and have contributed to the development of biotechnological tools that can be harnessed for control of CRISPR-Cas genome editing.

Introduction

All living organisms have viruses that infect them. In bacteria, these viruses are known as bacteriophages or phages. The estimated global population of phages is 4.80×10^{31} , and is considered the most abundant biological entity on the planet.¹ Phages are ubiquitous in nature and can be found wherever there are bacteria, resulting in bacteria constantly being at risk of phage infection. In fact, about 10^{23} phage infections occur every second,² placing enormous selective pressure on bacteria to fend off phages. As a result, bacteria have evolved a variety of innate anti-phage defense mechanisms, including those that are mediated at the cell surface, such as receptor masking and superinfection exclusion, and those that are mediated inside the cell, such as restriction modification and abortive infection mechanisms.³

In addition to these innate immunity mechanisms, bacteria have evolved an adaptive immune system known as the CRISPR-Cas system.⁴ CRISPR-Cas provides bacteria with robust protection against invasion by phages and other mobile genetic elements.^{5,6} However, phages have evolved ways to evade CRISPR-Cas defenses. These include mutation of the sequence in the phage ge-

nome that is targeted by the CRISPR-Cas system,⁷ and the activity of small proteins that directly interact with the CRISPR-Cas system and shut it down. These proteins are known as “anti-CRISPR” proteins. Since the initial report of their discovery in 2013,⁸ research on anti-CRISPR proteins has accelerated rapidly, revealing incredible genetic diversity and important implications for regulating CRISPR-Cas-mediated genome editing. This review covers the discovery of anti-CRISPR proteins, the current methods of identifying new anti-CRISPRs, and the diverse mechanisms by which they inhibit a variety of CRISPR-Cas systems. Additionally, the evolutionary impact of anti-CRISPRs on both bacteria and phages is also addressed.

The CRISPR-Cas Adaptive Immune System

The CRISPR-Cas system protects bacteria against foreign mobile genetic elements such as phages and plasmids.^{4,9} This adaptive immune system acquires protection against newly invading phages by integrating small fragments of the phage DNA called “spacers” into the CRISPR array.^{10–13} The CRISPR array acts as a record of phages that a bacterial cell has previously encountered. Much

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like our immune system, CRISPR-Cas is able to respond to future attack by these phages. To mediate immunity, the CRISPR locus is transcribed, and the transcript is processed to form short CRISPR RNAs (crRNAs), each containing a unique spacer sequence.^{14,15} The crRNA forms a ribonucleoprotein complex with the Cas proteins, known as the surveillance complex, which searches the cell for target sequences that match the spacer (protospacers).¹⁶ A short sequence known as the protospacer adjacent motif (PAM) found in many CRISPR-Cas systems allows the cell to differentiate between self-DNA and invading foreign genetic elements.^{7,10,17–19} Once the matching protospacer and PAM are detected, the target sequence is destroyed.

CRISPR-Cas systems exhibit tremendous diversity. Six types and more than 30 subtypes and variant subtypes of CRISPR-Cas systems have been identified to date.²⁰ While the distinct CRISPR-Cas systems differ in terms of their complement of *cas* genes and slight variations in mechanism, all of the systems fall under one of two broad classes. Class 1 systems (types I, III, and IV) utilize effector complexes formed by multiple Cas proteins, and class 2 systems (types II, V, and VI) use a large single effector protein that encodes all the activities needed for interference. Anti-CRISPR proteins have been identified for a variety of class 1 and class 2 CRISPR-Cas systems that span a wide range of bacteria.

The Discovery of Anti-CRISPR Proteins

Prior to the discovery of anti-CRISPR proteins, it was thought that the only way for phages to evade CRISPR-Cas immunity was by acquiring mutations. Point mutations in the PAM or seed sequence, a region of the protospacer that must have perfect complementarity to the crRNA, have been shown to be enough to escape the CRISPR-Cas system.^{7,21} However, because CRISPR-Cas systems are adaptive and can re-acquire new spacers, mutations alone are not sufficient for phages to avoid extinction.^{21,22} In 2013, the first active counter-mechanism to CRISPR-Cas immunity, anti-CRISPR proteins, was identified in phages that infect *Pseudomonas aeruginosa*.⁸ A number of *P. aeruginosa* strains, each with a unique phage genome incorporated as a prophage, were exposed to “CRISPR-sensitive” phages, which should be inhibited by the activity of the native type I-F CRISPR-Cas system. Remarkably, several of the prophage-containing strains allowed the CRISPR-sensitive phages to replicate. This suggested that these prophages encoded proteins that were able to interfere with CRISPR-Cas activity. Sequence analyses revealed a single genetic locus encoding 10 distinct protein families that was unique to the genomes of the *P. aeruginosa* prophages that mediated this anti-

CRISPR activity. Five of these genes encoded proteins that were shown to inhibit the type I-F CRISPR-Cas system in *P. aeruginosa* (AcrIF1–5; Table 1).⁸ Although the other genes in the anti-CRISPR locus did not have activity against the type I-F system, a follow-up study revealed that four of them inhibited the type I-E CRISPR-Cas system in *P. aeruginosa* (AcrIE1–4; Table 1).²³ Many *P. aeruginosa* phages have been shown to encode both type I-E and I-F anti-CRISPR genes, which would presumably provide them with an evolutionary advantage.

Probing for Additional Anti-CRISPRs

The nine anti-CRISPR protein families initially discovered were found exclusively within a small group of closely related *P. aeruginosa* phages. There were no identifiable homologues in other bacterial species or other types of mobile genetic elements. In addition, these anti-CRISPR proteins were extremely diverse in sequence and had no common motifs that could be used to probe for new anti-CRISPRs, confounding the search for additional anti-CRISPRs. To broaden the scope of known anti-CRISPR proteins, new search methods that combined bioinformatics and biochemical screens were developed. Several of these methods have been successful in uncovering novel anti-CRISPR proteins, yielding a total of 40 distinct families of anti-CRISPR proteins published to date (Table 1). The naming convention for anti-CRISPR genes and proteins, first established in 2015, includes reference to the subtype of CRISPR-Cas system that is inactivated. Inspired by the success of the CRISPR-Cas classification scheme, a database²⁴ was created to register new anti-CRISPR names to maintain order and avoid duplication. The most up to date list of anti-CRISPR proteins can be accessed there.

“Guilt-by-association” bioinformatics

The first approach to finding new anti-CRISPR proteins again arose from investigating *P. aeruginosa* phage genomes. Amazingly, although the initial nine anti-CRISPRs were not similar to each other, they all shared a highly conserved gene downstream of the anti-CRISPR genes. This conserved gene, named anti-CRISPR-associated gene 1 (*aca1*), encodes a predicted helix-turn-helix motif-containing protein. This motif is commonly found in transcriptional regulators.²⁵ BLAST searches using *Aca1* as a search query revealed homologues in a variety of prophage genomes across Proteobacteria. As the initial anti-CRISPR genes were encoded upstream of *aca1*, genes upstream of *aca1* homologues were cloned into expression plasmids and tested for the ability to inactivate the type I-E and I-F CRISPR-Cas systems of *P. aeruginosa*. This led to the identification of five new families of anti-CRISPR

Table 1. Anti-CRISPR Protein Families Identified to Date

Family	Origin of characterized member	Accession no.	CRISPR-Cas system inhibited (organism)	Ref.
AcrIC1	<i>Moraxella bovoculi</i> prophage	AKG19229.1	I-C (Pae)	30
AcrID1	<i>Sulfolobus islandicus</i> rudivirus 3	YP_009272954.1	I-D (Sis)	39
AcrIE1	<i>Pseudomonas aeruginosa</i> phage JBD5	YP_007392738.1	I-E (Pae)	23, 47
AcrIE2	<i>P. aeruginosa</i> phage JBD88a	YP_007392439.1	I-E (Pae)	23
AcrIE3	<i>P. aeruginosa</i> phage DMS3	YP_950454.1	I-E (Pae)	23
AcrIE4	<i>P. aeruginosa</i> phage D3112	NP_938238.1	I-E (Pae)	23
AcrIE4-F7	<i>Pseudomonas citronellolis</i> prophage	WP_064584002.1	I-E/I-F (Pae)	30
AcrIE5	<i>Pseudomonas otitidis</i> prophage	WP_074973300.1	I-E (Pae)	30
AcrIE6	<i>P. aeruginosa</i> prophage	WP_087937214.1	I-E (Pae)	30
AcrIE7	<i>P. aeruginosa</i> prophage	WP_087937215.1	I-E (Pae)	30
AcrIF1	<i>P. aeruginosa</i> phage JBD30	YP_007392342.1	I-F (Pae, Pec)	8, 26, 40, 41, 42, 43, 52
AcrIF2	<i>P. aeruginosa</i> phage D3112	NP_938237	I-F (Pae, Pec)	8, 26, 40, 41, 42, 43, 53
AcrIF3	<i>P. aeruginosa</i> phage JBD5	YP_007392440.1	I-F (Pae)	8, 26, 40, 48, 49
AcrIF4	<i>P. aeruginosa</i> phage JBD26	WP_016068584.1	I-F (Pae)	8, 26, 40
AcrIF5	<i>P. aeruginosa</i> phage JBD5	YP_007392740.1	I-F (Pae)	8, 40
AcrIF6	<i>P. aeruginosa</i> prophage	WP_043884810	I-E (Pae)/I-F (Pae, Pec)	26
AcrIF7	<i>P. aeruginosa</i> prophage	ACD38920.1	I-F (Pae, Pec)	26
AcrIF8	<i>Pectobacterium</i> phage ZF40	AFC22483.1	I-F (Pae, Pec)	26
AcrIF9	<i>Vibrio parahaemolyticus</i> mobile element	WP_031500045.1	I-F (Pae, Pec)	26
AcrIF10	<i>Shewanella xiamenensis</i> prophage	KEK29119	I-F (Pae, Pec)	26, 42
AcrIF11	<i>P. aeruginosa</i> prophage	WP_038819808.1	I-F (Pae)	30
AcrIF12	<i>P. aeruginosa</i> mobile element	ABR13388.1	I-F (Pae)	30
AcrIF13	<i>Moraxella catarrhalis</i> prophage	EGE18854.1	I-F (Pae)	30
AcrIF14	<i>Moraxella</i> phage Mcat5	AKI27193.1	I-F (Pae)	30
AcrIIA1	<i>Listeria monocytogenes</i> prophage J0161a	WP_003722518.1	II-A (Lmo)	34, 54
AcrIIA2	<i>L. monocytogenes</i> prophage J0161a	WP_003722517.1	II-A (Lmo, Spy)	34, 44, 45, 46
AcrIIA3	<i>L. monocytogenes</i> prophage SLCC2482	WP_014930691.1	II-A (Lmo)	34
AcrIIA4	<i>L. monocytogenes</i> prophage J0161b	WP_003723290.1	II-A (Lmo, Spy)	34, 46, 55, 56, 57
AcrIIA5	<i>Streptococcus thermophilus</i> phage D4276	ASD50988.1	II-A (Sth, Spy)	37, 38
AcrIIA6	<i>S. thermophilus</i> phage D1811	AVO22749	II-A (Sth)	38
AcrIIC1	<i>Neisseria meningitidis</i>	WP_049360089.1	II-C (Nme, Cje, Geo, Hpa, Smu)	27, 28, 29
AcrIIC2	<i>N. meningitidis</i> prophage	WP_042743678.1	II-C (Nme, Hpa, Smu)	27, 29
AcrIIC3	<i>N. meningitidis</i> prophage	WP_042743676.1	II-C (Nme, Hpa, Smu)	27, 28, 29
AcrIIC4	<i>Haemophilus parainfluenzae</i> prophage	WP_049372635.1	II-C (Nme, Hpa, Smu)	29
AcrIIC5	<i>Simonsiella muelleri</i> prophage	WP_002642161.1	II-C (Nme, Hpa, Smu)	29
AcrVA1	<i>M. bovoculi</i> prophage	AKG19227.1	V-A (Mb, As, Lb, Fn)	30, 35
AcrVA2	<i>M. bovoculi</i> prophage	AKG19228.1	V-A (Mb)	30
AcrVA3	<i>M. bovoculi</i> prophage	AKG19230.1	V-A (Mb)	30
AcrVA4	<i>M. bovoculi</i> mobile element	WP_046699156.1	V-A (Mb, Lb)	35
AcrVA5	<i>M. bovoculi</i> mobile element	WP_046699157.1	V-A (Mb, Lb)	35

As, *Acidaminococcus* sp.; Cje, *Campylobacter jejuni*; Fn, *Francisella novicida*; Geo, *Geobacillus stearothermophilus*; Hpa, *Haemophilus parainfluenzae*; Lb, *Lachnospiraceae bacterium*; Lmo, *Listeria monocytogenes*; Mb, *Moraxella bovoculi*; Nme, *Neisseria meningitidis*; Pae, *Pseudomonas aeruginosa*; Pec, *Pectobacterium atrosepticum*; Sis, *Sulfolobus islandicus*; Spy, *Streptococcus pyogenes*; Sth, *Streptococcus thermophilus*.

proteins with activity against the type I-F system (AcrIF6-10; Table 1).²⁶ One of these newly identified type I-F anti-CRISPRs was also found to inactivate the type I-E CRISPR-Cas system. This was the first anti-CRISPR shown to inhibit CRISPR-Cas across multiple subtypes.

This “guilt-by-association” method of BLAST searches using *aca1* subsequently led to the discovery of the first anti-CRISPRs against a type II CRISPR-Cas system, which uses Cas9 as its effector protein. It was observed that some of the homologues of these newly discovered type I-F anti-CRISPRs were not associated with *aca1*, but instead were associated with a different helix-turn-helix containing protein that was designated *aca2*. Further bioinformatic analyses using these *aca2* proteins as a starting point identified three anti-CRISPR proteins with activity against the type II-C system in *Neisseria meningitidis*

(AcrIIC1-3; Table 1).²⁷ AcrIIC1 was shown to inhibit a range of type II-C CRISPR-Cas systems, including Cas9 from *Campylobacter jejuni*, *Geobacillus stearothermophilus*, *Haemophilus parainfluenzae*, and *Simonsiella muelleri*.^{28,29} These anti-CRISPRs were also verified to inhibit Cas9 in cultured human cells, making them the first examples of anti-CRISPR proteins being used as off switches of Cas9-mediated genome editing.²⁷ Another study found two additional type II-C anti-CRISPRs encoded upstream of *aca2* in *H. parainfluenzae* and *S. muelleri* prophage genomes (AcrIIC4,5; Table 1).²⁹ AcrIIC5 is currently the most potent anti-CRISPR against *Neisseria meningitidis* Cas9 (NmeCas9).²⁹

Years after the initial discovery of *aca1*, as more bacterial and phage sequence data were compiled, BLAST searches using *aca1* as a search query yielded new

genes that had not yet been tested for anti-CRISPR activity. Functional assays of these genes revealed three new type I-E anti-CRISPRs (AcrIE5–7; Table 1), one new type I-F anti-CRISPR (AcrIF11; Table 1), and a chimera (AcrIE4-F7; Table 1) that had activity against both the type I-E and I-F CRISPR-Cas systems.³⁰ This study also uncovered new families of anti-CRISPR-associated genes, *aca4* through *aca7*, and another type I-F anti-CRISPR (AcrIF12; Table 1) associated with *aca4*. Homologs of the newly identified type I-F anti-CRISPR, *acrIF11*, were found in more than 50 species of Proteobacteria. In an attempt to find inhibitors against CRISPR-Cas systems without any known anti-CRISPR proteins, genes adjacent to *acrIF11* were investigated in *Moraxella* strains, which harbor the type I-C, I-F, and V-A CRISPR-Cas systems. As a result, two more anti-CRISPRs for the type I-F system were found (AcrIF13,14; Table 1), and the first anti-CRISPRs against the type I-C and V-A systems were discovered (AcrIC1, AcrVA1–3; Table 1).³⁰ AcrVA1–3 were tested against various Cas12a orthologs, with AcrVA1 showing activity against a broad spectrum of Cas12a proteins from *Moraxella bovoculi*, *Acidaminococcus* sp., *Lachnospiraceae* bacterium, and *Francisella novicida*.³⁰ Other type V-A anti-CRISPRs were simultaneously discovered using a different bioinformatic approach as discussed below. The discovery of type V-A anti-CRISPR proteins was an exciting advance, as Cas12a, the effector protein in type V-A CRISPR-Cas systems, is an attractive alternative to Cas9 for genome editing purposes.^{31–33}

Self-targeting spacers

A different bioinformatic approach based on the identification of self-targeting spacers in bacteria was developed to find novel anti-CRISPRs in diverse CRISPR-Cas systems. The idea was that there must be a CRISPR-Cas inhibitor that prevents the bacterium from killing itself when it possesses a CRISPR spacer that targets its own genome. Several *Listeria monocytogenes* isolates, which encode type II-A CRISPR-Cas systems, were found to have self-targeting spacers. Many of these spacers targeted prophage sequences, whose genes were investigated for anti-CRISPR activity. Using this approach, four families of anti-CRISPR proteins that inhibit the type II-A Cas9 in *L. monocytogenes* were discovered (AcrIIA1–4; Table 1).³⁴ Of these four, AcrIIA2 and AcrIIA4 were also active against the type II-A *Streptococcus pyogenes* Cas9 (SpyCas9), the most commonly used Cas9 in genome editing.

Cell-free transcription-translation functional screen

Recently, the self-targeting bioinformatics approach was coupled with a systematic functional screen to generate a

novel screening pipeline for finding new anti-CRISPRs. First, the Self-Targeting Spacer Search (STSS) was used to predict self-targeting spacers in all available bacterial genomes with CRISPR-Cas systems. In an attempt to finding novel type V-A anti-CRISPRs, the STSS results were narrowed down to self-targeting *Moraxella bovoculi* strains. Overlapping ~2–10 kb fragments of predicted mobile genetic elements from three *M. bovoculi* genomes were generated by polymerase chain reaction.³⁵ The presence of anti-CRISPR activities encoded in these genomic fragments was tested using a cell-free transcription-translation (TXTL) system, a method first utilized for anti-CRISPR proteins by Marshall *et al.*³⁶ Using the TXTL system, a fully functional type V-A CRISPR-Cas system was reconstituted *in vitro*. Cas12a activity was measured using a reporter plasmid encoding green or red fluorescent protein. The plasmid was targeted by the CRISPR-Cas12a system, so active Cas12a led to a decrease in fluorescence, while fluorescence was restored if the genomic fragment encoded a Cas12a inhibitor. Of 67 genomic fragments tested, four provided increased fluorescence. The open reading frames of the four genomic fragments were individually cloned and tested using the TXTL Cas12a assay, and three genes were determined to have anti-CRISPR activity against Cas12a (*acrVA1*, *acrVA4*, and *acrVA5*; Table 1).³⁵ Interestingly, AcrVA1 was discovered concurrently using two different methods.^{30,35}

“Phage-first” approach

Some anti-CRISPR proteins were discovered by harnessing phages themselves, such as AcrIIA5 and AcrIIA6 (Table 1), which were identified in virulent *Streptococcus thermophilus* phages.^{37,38} Five *S. thermophilus* phages were initially investigated for possible anti-CRISPR activity. Strains of *S. thermophilus* with an active type II-A CRISPR-Cas system were challenged with these phages. While the *S. thermophilus* strains were able to develop CRISPR immunity against four of the phages, the bacteria could not protect themselves against phage D4276.³⁷ Each gene of the D4276 genome was cloned onto a plasmid and expressed in *S. thermophilus*. A CRISPR-sensitive phage was then plated on the bacteria to see if the phage titer would be restored by the activity of an anti-CRISPR gene. In doing so, *acrIIA5* was discovered (Table 1), making it the first anti-CRISPR to be identified in a virulent phage.³⁷ Quickly following was the discovery of AcrIIA6, which was found in another virulent *S. thermophilus* phage D1811 (Table 1).³⁸ AcrIIA5 demonstrates a broad activity range as it inhibits both *S. pyogenes* and *S. thermophilus* Cas9 proteins, which share only 25% sequence identity. Even more striking, an

in vitro study suggests that AcrIIA5 may be able to also inhibit type II-C Cas9 proteins.³⁶

A similar “phage-first” approach was used to find the first archaeal virus anti-CRISPR. *Sulfolobus islandicus*, an archaeal strain that harbors type I-A, I-D, and III-B CRISPR-Cas systems, is predicted to have spacers that should protect against the virus SIRV2. However, this virus was able to infect this strain readily. Interestingly, a SIRV2 mutant (SIRV2M) that lost a 4 kb fragment after successive infections in *Sulfolobus solfataricus*, a CRISPR-less archaeon, was not able to infect *S. islandicus*. In this 4 kb fragment, several genes were found to be conserved in another virus, known as SIRV3, which can also infect *S. islandicus*. These conserved genes were cloned individually from SIRV3 into *S. islandicus* before infecting with SIRV2M. One gene was found to be necessary and sufficient to restore the infectivity of SIRV2M in *S. islandicus*. This gene was shown to inhibit the type I-D CRISPR-Cas system in *S. islandicus* by directly interacting with the Cas10d subunit, thus being named *acrID1* (Table 1).³⁹ AcrID1 was the first inhibitor found for type

I-D systems, and the first anti-CRISPR found in an archaeal virus.

Diverse Mechanisms of Anti-CRISPR Proteins

To date, 40 distinct families of anti-CRISPR proteins have been reported (Table 1). Anti-CRISPRs are incredibly diverse, sharing no common sequence or structural motifs. They inhibit a wide range of CRISPR-Cas systems, both class 1 and class 2, and have various mechanisms of inhibition (Fig. 1). There are multiple steps of CRISPR-Cas activity that can be targeted by anti-CRISPR proteins. CRISPR-Cas immunity can be broken down into three broad stages: spacer acquisition, crRNA biogenesis, and foreign DNA interference. Although all anti-CRISPRs with elucidated mechanisms have been shown to obstruct the interference stage, the other stages can theoretically be inhibited to shut down the CRISPR-Cas system. For instance, anti-CRISPRs may be able to prevent Cas proteins from acquiring new spacers. They could potentially also block transcription of the CRISPR array or processing of the transcript into mature crRNAs.

FIG. 1. Anti-CRISPR proteins inhibit type I and II CRISPR-Cas systems at different stages. **(A)** In type I CRISPR-Cas systems, the Cas5-Cas8 heterodimer, Cas6, and six subunits of Cas7 come together with the crRNA to form the surveillance complex. The complex then uses the spacer sequence (yellow) to survey the cell and binds to target DNA with the corresponding protospacer (red). The Cas3 nuclease is recruited to the complex and cleaves the target DNA. AcrIF1, AcrIF2, and AcrIF10 inhibit the target DNA binding stage. AcrIE1 and AcrIF3 block Cas3 recruitment and thereby prevent DNA cleavage. **(B)** The loading of crRNA onto Cas9 initiates type II CRISPR-Cas systems. Cas9 then binds to target DNA, which is later cleaved by nuclease domains of Cas9. AcrIIA2, AcrIIA4, AcrIIC3, AcrIIC4, and AcrIIC5 are known to prevent target DNA binding by Cas9. AcrIIC1 inhibits Cas9 from cleaving the target DNA.

The anti-CRISPR proteins that inhibit the interference stage bind directly to the effector protein complex and could thereby inhibit one of three steps: crRNA loading, target DNA binding, or target DNA cleavage. Anti-CRISPRs that obstruct crRNA loading have not yet been reported, but anti-CRISPRs that block both target DNA binding and cleavage have.

Blocking DNA binding

Eleven anti-CRISPR proteins have known mechanisms, and eight have been shown to block DNA binding, making this the most common mechanism of inhibition by anti-CRISPRs. Interestingly, anti-CRISPRs inhibit DNA binding by various means. AcrIF1, AcrIF2, and AcrIF10 all inhibit the type I-F CRISPR-Cas system by preventing DNA binding to the Cascade effector complex. However, they do this by different mechanisms. AcrIF1 was shown, by both size-exclusion chromatography and cryo-electron microscopy, to bind the Cas7f backbone of the Cascade complex.^{40–43} Structural analysis has shown that AcrIF1 induces a conformational change in the Cascade complex that prevents the target DNA from accessing and hybridizing with the crRNA.⁴¹ AcrIF2, on the other hand, wedges itself between the Cas7f subunit located at the tail end of the backbone and Cas8f, thereby blocking target recognition through competition for a critical DNA binding site.^{40,41,43} AcrIF10 binds to a DNA binding site formed by Cas8f and Cas5f, forcing the Cascade complex to adopt the DNA-bound conformation.⁴²

Anti-CRISPR proteins that act against type II CRISPR-Cas systems have also been shown to act by blocking DNA binding. Using cryo-electron microscopy, the type II-C anti-CRISPR protein AcrIIC3 was shown to dimerize Type II-C Cas9 and thereby prevent DNA binding.²⁸ Electrophoretic mobility shift assay experiments revealed that both AcrIIC4 and AcrIIC5 prevent NmeCas9 from binding to DNA while still allowing sgRNA loading.²⁹ Both cryo-electron microscopy and X-ray crystallography structures demonstrated that AcrIIA2 acts as a DNA mimic and occupies the DNA binding site on Cas9.^{44,45} A crystal structure of AcrIIA4-bound SpyCas9 revealed that AcrIIA4 binds to the PAM-interacting domain, thus preventing the binding and probing of the PAM sequence on the target DNA.⁴⁶

Preventing DNA cleavage

Three anti-CRISPR proteins have been shown to inhibit CRISPR-Cas by preventing target DNA cleavage. The type I-E anti-CRISPR AcrIE1 has been shown to bind and inhibit Cas3, the nuclease protein in type I CRISPR-Cas systems.⁴⁷ AcrIF3 also binds directly to Cas3 and

prevents the recruitment of Cas3 to the Cascade complex.⁴⁰ X-ray crystallography and cryo-electron microscopy structures revealed that AcrIF3 binds to Cas3 as a dimer so that the binding sites of Cas3 to the Cascade complex and DNA are blocked.^{48,49} AcrIIC1 is the lone anti-CRISPR shown to prevent DNA cleavage by type II-C Cas9s. AcrIIC1 was shown to bind directly to the HNH endonuclease domain of Cas9 using both biochemical and structural studies.²⁸ This binding prevents DNA cleavage but still allows the target DNA to bind to Cas9, essentially turning the Cas9 into dCas9.²⁸

Countering Anti-CRISPRs: The Evolutionary Arms Race

The ongoing evolutionary arms race between bacteria and phages suggests that bacteria may have evolved ways to counter anti-CRISPR proteins, just as phages evolved anti-CRISPRs to fight against the CRISPR-Cas system. One obvious way bacteria evade anti-CRISPR inhibition is to harbor multiple types of CRISPR-Cas systems, as seen in *Pseudomonas* and *Moraxella* species. By having several active CRISPR-Cas systems, bacteria can protect themselves from phages that have anti-CRISPRs for a single type of CRISPR-Cas system. However, phages may also respond to this evolutionary pressure. For example, anti-CRISPR protein AcrIF6 has dual specificity and is able to inhibit both type I-E and I-F CRISPR-Cas systems.²⁶ Another way bacteria can evade anti-CRISPR activity is by amassing mutations in the Cas proteins so that they escape targeting by the anti-CRISPR proteins. In fact, the Cascade complex of a type I-Fv (type I-F variant) CRISPR-Cas system of *Shewanella putrefaciens* CN32 was characterized by X-ray crystallography and was found to differ significantly from the prototypical type I-F Cascade complex at the known anti-CRISPR binding sites.⁵⁰ This suggests that this bacterial species may have evolved its Cas proteins to evade inhibition by type I-F anti-CRISPRs. One way in which anti-CRISPRs can combat this evasion is through interactions with critical residues in the Cas proteins. This has been shown in the case of AcrIIC1, which interacts with the active-site residues in the HNH endonuclease domain of CRISPR-Cas9.²⁸ Thus, if the bacterial cell mutates residues to evade anti-CRISPR binding, it will also lose the ability to cleave the invading DNA. Other ways by which bacteria might be able to counter anti-CRISPR defenses include silencing expression of anti-CRISPR genes or targeting anti-CRISPR proteins for proteolysis. Bacteria could also arm themselves with “anti-anti-CRISPRs” to inactivate anti-CRISPRs before the CRISPR-Cas system is inhibited.⁵¹ However, evidence of these counter mechanisms has not been found to date.

Outlook for Anti-CRISPR Research

Since the discovery of the first anti-CRISPRs in 2013, the pace of identification of new families has rapidly increased, leading to the current total of 40 distinct families of anti-CRISPR protein families (Table 1). A variety of bioinformatic and functional assays have been developed to expedite anti-CRISPR discovery. However, most CRISPR-Cas systems still have no known inhibitors, suggesting that many anti-CRISPR protein families are yet to be discovered. Much work remains to be done characterizing the identified anti-CRISPR families, as only 11 of the 40 anti-CRISPR families have known mechanisms of activity. Determining these mechanisms will support the development of anti-CRISPRs as regulators for genome engineering and other applications. The variety of mechanisms by which anti-CRISPRs act is broad, and some mechanisms may be more favorable for specific applications. In addition, elucidating the diversity of anti-CRISPR mechanisms will further our understanding of how phages and bacteria compete in the ongoing evolutionary arms race. Studies of phage–host interactions have yielded important biotechnological tools, including restriction enzymes, phage therapy, and, of course, CRISPR-Cas. Now, phage-encoded anti-CRISPRs provide potential regulators of CRISPR-Cas activity that could be used for a variety of biotechnological applications. Continued studies of phage and bacterial interactions will undoubtedly lead to the development of useful new technologies.

Author Disclosure Statement

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