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Inhibition of CRISPR-Cas Systems by Mobile Genetic Elements

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Abstract

Clustered, regularly interspaced, short, palindromic repeats (CRISPR) loci, together with their CRISPR-associated (Cas) proteins, provide bacteria and archaea with adaptive immunity against invasion by bacteriophages, plasmids, and other mobile genetic elements. These host defenses impart selective pressure on phages and mobile elements to evolve countermeasures against CRISPR immunity. As a consequence of this pressure, phages and mobile elements have evolved “anti-CRISPR” proteins that function as direct inhibitors of diverse CRISPR-Cas effector complexes. Some of these CRISPR-Cas complexes can be deployed as genome engineering platforms, and anti-CRISPRs could therefore be useful in exerting spatial, temporal, or conditional control over genome editing and related applications. Here we describe the discovery of anti-CRISPRs, the range of CRISPR-Cas systems that they inhibit, their mechanisms of action, and their potential utility in biotechnology.

Introduction

Microbes and phages have been engaging in co-evolutionary battle for billions of years, almost certainly since very shortly after the dawn of cellular life. It is estimated that $\sim 10^{30}$ bacterial or archaeal cells are attacked by viruses each and every day throughout earth's biosphere [1]. Bacteria flourish even in the face of such carnage, suggesting that many of those infections do not succeed, thanks in part to defensive strategies that prevent or interrupt them. Some forms of host defense have been recognized for many years, including strategies based on surface exclusion, abortive infection, and restriction-modification [2]. Not surprisingly, phages have responded by evolving myriad strategies to thwart or circumvent these defenses.

More recently, microbiologists have come to recognize another widespread collection of bacterial and archaeal defense pathways based on CRISPR loci [3–6]. The common, basic strategy of CRISPR-Cas pathways (reviewed in [7,8]) begins with the incorporation of a small piece of an invasive genome [usually from a phage, but sometimes from a plasmid or

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other mobile genetic element (MGE)] as a “spacer” in a CRISPR array. The spacer, along with flanking CRISPR repeat sequence, is expressed as a small CRISPR RNA (crRNA) that loads into an effector complex [comprised of one or more Cas proteins] harboring latent nuclease activity. On its own, the Cas effector lacks the capacity to recognize and degrade invasive nucleic acids. However, the crRNA functions as a specificity factor, allowing invasive sequences complementary to the spacer to be recognized via Watson-Crick base pairing, leading to Cas nuclease activation and invader destruction. Spacer acquisition thereby enables hosts to record exposure to previous attacks, to use that genomic record to confer adaptive immunity to future attacks by viruses and MGEs with similar sequences, and to pass these genomic records to their progeny. Many mechanistic, physiological, and evolutionary aspects of CRISPR interference are described in detail in other contributions in this volume.

As with the challenges that phages and MGEs have faced from surface exclusion, abortive infection, and restriction-modification host defenses, CRISPR interference has driven the evolution of countermeasures that enable phage survival and MGE dissemination. The requirement for sequence complementarity between crRNA and phage/MGE [as well as other target sequence requirements such as the “protospacer adjacent motif” (PAM) that licenses interference] suggests that phages and MGEs can often exploit genetic variation to evade CRISPR interference, and it was established very early that this is indeed the case [9,10]. However, phages and MGEs have also arrived at more active countermeasures in the form of anti-CRISPR (Acr) proteins, which can disarm CRISPR defenses for any target sequence, including those that are functionally constrained from sequence drift. The ability of CRISPR systems to drive phages to extinction in spite of rapid mutational evasion [11] suggests that anti-CRISPRs can be indispensable for long-term phage survival [12].

CRISPR-Cas Systems Subject to Anti-CRISPR Inhibition

CRISPR-Cas systems are remarkably diverse, with at least six “types,” each of which is further divided into multiple subtypes [13]. The CRISPR-Cas types are primarily defined by their distinct effector machineries, with substantial differences in targeting mechanisms between them. Types I and III are the most widespread in nature [13], and both employ large, multisubunit effector complexes [7,8]. Type II is the next most abundant, and uses a single Cas protein (Cas9) as an effector [14]. Types I and II recognize and degrade DNA, whereas Type III systems naturally target both RNA and DNA for destruction [7,8]. Types IV, V and VI are more recently discovered, less broadly distributed, and less well understood [13,15]. Type III, IV, V and VI CRISPR-Cas systems are not yet known to be subject to inhibition by anti-CRISPRs and will not be considered further here.

In type I CRISPR-Cas systems, target recognition and degradation activities are physically separable: crRNA target recognition is done by a multisubunit complex [4], which then recruits an extrinsic nuclease (Cas3) for target destruction (Fig. 1a) [16]. Six subtypes (I-A through I-F) have been defined [13]. In type II, Cas9 associates not only with a crRNA, but also with a second small RNA called a tracrRNA that is partially complementary to (and annealed with) the crRNA’s repeat-derived region (Fig. 1b) [17,18]. The Cas9-crRNA-tracrRNA complex performs both primary effector functions (target recognition and

cleavage) (Fig. 1b) [18,19]. Type II systems are subdivided into three subtypes (II-A, II-B and II-C), mostly based on Cas9 sequence and architecture [13,15]. Despite their complete lack of molecular relatedness, type I and type II systems share certain functional features: both require a PAM for target engagement (Fig. 1) [7,8], and crRNAs for both exhibit PAM-proximal “seed” regions with heightened intolerance for mismatches with their targets [20]. PAM and seed polymorphisms are among the most common means of mutational evasion of CRISPR interference by phages and MGEs that are confronted by type I and type II systems [9–11,21].

Type I Anti-CRISPRs

Early explorations of type I CRISPR interference employed several experimental models, including a Type I-F system in *Pseudomonas aeruginosa* strain PA14 (Fig. 1a). Numerous prophages have been defined in *P. aeruginosa*, and in many cases, lysogens prevent subsequent infection by other phages (superinfection exclusion). However, in some cases, lysogeny was found to *potentiate* infection by certain other lytic phages. Subsequent experiments revealed the explanation for this unexpected finding: in the wild-type (non-lysogenized) strain, the Type I-F CRISPR-Cas system inhibits lytic phage infection (due to phage-matched CRISPR spacers), but in the otherwise isogenic lysogen, CRISPR interference is inhibited by genes expressed from the prophage. The basis for this inhibition proved to be a set of small open reading frames (depending on the lysogen) encoding proteins with no detectable similarities to other proteins. These anti-CRISPR (Acr) proteins fell into five distinct families. In all cases their inhibitory functions were specific to subtype I-F, as they had no effect on the type I-E CRISPR-Cas system of *E. coli* or *P. aeruginosa* [22]. A subsequent study revealed that the same phage operons encoding the type I- F anti-CRISPRs also encoded inhibitors of the *P. aeruginosa* type I-E system [23].

Anti-CRISPR Mechanisms

A remarkable feature of anti-CRISPRs is their uniformly small size (~50–150 amino acids) and their lack of sequence similarity to any proteins of known function. Thus, only experimental studies could supply insight into how these proteins function. The initial study on anti-CRISPR mechanism employed *in vitro* biochemical approaches to show that 3 type I-F anti-CRISPRs each inhibited the *P. aeruginosa* system in a different way [24]. Two of these anti-CRISPRs, AcrF1 and AcrF2, bound directly to the Csy complex (the subtype I-F Cas protein/crRNA complex that recognizes DNA targets) and abrogated DNA-binding activity (Fig. 2a). However, they bound different subunits of the complex. The third anti-CRISPR, AcrF3, inhibited CRISPR-Cas function by interacting with the Cas3 nuclease and preventing its recruitment to the DNA-bound Csy complex (Fig. 2a). An interesting feature of the AcrF3 mechanism is that Csy complex binding to DNA still occurs. Thus, targeting of the Csy complex to a transcriptional promoter in the presence of AcrF3 leads to transcriptional repression [24].

High-resolution structural studies have elucidated the interactions of anti-CRISPRs with CRISPR-Cas components. A large (2,500 Å²) interaction interface was observed between Cas3 and an AcrF3 dimer [25,26]. In this complex, the Cas3 binding sites for both the Csy

complex and DNA are blocked, and it is locked into an ADP-bound inactive form; thus Cas3 function is stifled in every possible way. A very exciting 3.4 Å resolution cryoEM structure of the *P. aeruginosa* type I-F Csy complex bound to two anti-CRISPRs, AcrF1 and AcrF2, was recently published [27]. Two molecules of AcrF1 were seen to bind at the intersubunit interfaces of Cas7f, six copies of which form the helical backbone of the Csy complex. Binding of AcrF1 likely alters the conformation of a flexible region of Cas7f causing occlusion of the DNA-binding site. AcrF1 also directly interacts with residues that appear to be important for DNA-binding. The structure of AcrF1 determined by cryoEM matched a previously solved NMR structure [28], and key functional residues identified through mutagenesis in this study interact closely with residues in Cas7f. AcrF2 was shown to bind a portion of Cas8f involved in DNA-binding activity [27]. Interestingly, the charge distribution of the surface of AcrF2 resembles B-form dsDNA, suggesting that this anti-CRISPR acts as a DNA mimic.

Identifying anti-CRISPRs in diverse bacterial species

The initial nine families of anti-CRISPRs were all found within one operon in a group of closely related phages infecting *P. aeruginosa* strain PA14 [22,23]. Although each of these families was entirely distinct, sequence-based searches with any of these anti-CRISPRs revealed no homologues outside of the *Pseudomonas* genus. However, all of the anti-CRISPR operons in these phages also included a highly conserved gene, referred to as *aca1*, that did not possess anti-CRISPR activity and appears to be a transcriptional regulator [29]. By performing BLAST searches with the Aca1 protein and examining the proteins encoded by genes adjacent to *aca1* orthologues, it was possible to identify five new families of type I-F anti-CRISPRs and a new family of Aca proteins (Aca2) [29]. These new families of anti-CRISPRs included members of diverse bacterial species spanning the full diversity of Proteobacterial species in which type I-F CRISPR-Cas systems are found. Furthermore, through bioinformatic analysis and testing against two type I-F CRISPR-Cas systems, many of these anti-CRISPRs were shown to possess broad specificity [29]. It was concluded that anti-CRISPRs exist that would inhibit any of the known type I-F CRISPR-Cas systems. Type I anti-CRISPRs reported to date are listed in Table 1.

Type II Anti-CRISPRs

Searches for anti-CRISPRs using known, type-specific Acr sequences as queries have limited potential to identify other Acrs outside of the same CRISPR-Cas type. However, the recognition that *aca* genes encode likely Acr transcriptional control proteins [29] raised the possibility of a distinct strategy (searches for uncharacterized ORFs adjacent to *aca* orthologs) with greater potential to cross the boundaries of CRISPR-Cas systems (Fig. 3a), since the Aca proteins need not necessarily engage any specific CRISPR-Cas system components directly. An *aca2* ortholog (with a small, novel ORF upstream of it) was indeed identified in a putative MGE in a strain of *Brackiella oedipodis* that harbored a Type II-C CRISPR-*cas* locus but no Type I system [30]. The adjacent candidate *acr* gene had an ortholog in a strain of *Neisseria meningitidis* that was adjacent to a distinct candidate *aca* gene (*aca3*). The jump from *B. oedipodis* to *N. meningitidis* was especially significant because numerous meningococcal strains have been identified with Type II-C CRISPR-Cas

systems, and one such system (in strain 8013) was functionally validated, developed as a II-C prototype [31,32], and successfully adapted for NmeCas9 genome editing applications [33–35]. Yet another meningococcal strain encoded a different *aca3* ortholog adjacent to two more novel ORFs. Thus, using the strategy depicted in Fig. 3a, several candidate Type II-C anti-CRISPRs were compiled, three of which were in a species readily amenable to functional analysis. Tests of native CRISPR interference in *N. meningitidis* 8013 validated all three meningococcal candidates (AcrIIC1_{Nme}, AcrIIC2_{Nme}, and AcrIIC3_{Nme}) as bona fide Type II-C anti-CRISPRs [30]. Intriguingly, the *B. oedipodis* candidate (AcrIIC1_{Boe}) was also found to prevent interference by the native NmeCas9 (~47% identical to *B. oedipodis* Cas9), indicating cross-species inhibition and suggesting that some type II anti-CRISPRs could be broad-spectrum Cas9 inhibitors. Purified, recombinant AcrIIC1_{Nme}, AcrIIC2_{Nme}, and AcrIIC3_{Nme} were each found to bind to sgRNA-loaded NmeCas9 directly *in vitro*, and to inhibit its DNA cleavage activity (AcrIIC1_{Boe} was not tested *in vitro*). Importantly for technological development, all four Acrs were found to reduce or eliminate genome editing activity in human cells, indicating that the Acrs can be deployed as off-switches to control the spatial, temporal or conditional extent of editing. Finally, AcrIIC3_{Nme} was shown to prevent DNA binding by “dead” NmeCas9 (dNmeCas9) in human cells, revealing its potential to control dNmeCas9-effector fusion applications [36–38] such as CRISPRi/CRISPRa [39,40].

A distinct approach recently yielded additional, unique Cas9 inhibitors. Many functional CRISPR-Cas systems (including those from Type II) impart an incompatibility between CRISPR spacers and cognate protospacers (including within integrated prophages) within the same genome, due to the cell death that arises from “autoimmune” targeting and destruction of the host chromosome. However, the expression of an anti-CRISPR could prevent lethality in strains that acquire matched spacer/protospacer pairs, because the otherwise lethal self-targeting event is inhibited (Fig. 3b). Rauch *et al.* [41] searched for genome-sequenced, Cas9-containing strains with unusually high frequencies of apparent self-targeting, hypothesizing that these genomes could harbor prophages expressing candidate anti-CRISPRs. This scenario was confirmed to exist in certain strains of *Listeria monocytogenes* that encode Type II-A CRISPR-Cas systems [41]. Subsequent bioinformatics searches and genetic tests identified four prophage-encoded anti-CRISPR genes (*acrIIA1-4*), each of which sufficed for inhibition of CRISPR interference by *L. monocytogenes* Cas9 (LmoCas9). Importantly, two of the validated anti-CRISPRs (AcrIIA2_{Lmo} and AcrIIA4_{Lmo}) exhibited weak or strong inhibition, respectively, of genome editing catalyzed by *Streptococcus pyogenes* Cas9 (SpyCas9), which is by far the most frequently used Cas9 ortholog for genome editing applications [42,43]. SpyCas9 and LmoCas9 are 53% identical at the amino acid sequence level, again indicating that this degree of similarity can suffice for cross-species inhibition by a single Acr protein, and reinforcing the likelihood of broad-spectrum Cas9 inhibition by some type II anti-CRISPRs. Finally, both AcrIIA2_{Lmo} and AcrIIA4_{Lmo} were shown to inhibit transcriptional repression by promoter-directed dSpyCas9 in *E. coli* [41], indicating that both Acrs prevent stable, sgRNA-guided DNA binding by dSpyCas9. Three recent reports describe the structure of AcrIIA4_{Lmo} in complex with sgRNA-loaded SpyCas9, revealing a DNA-mimicking inhibitor that binds to the PAM-interacting region of the SpyCas9/sgRNA complex [44–46],

reminiscent of the inhibitory logic exhibited by the type I inhibitor AcrF2 (see above). In agreement with the functional tests of Rauch *et al.* [41], AcrIIA4_{Lmo} binding prevents the engagement of sgRNA-complementary target DNA by SpyCas9. These observations establish the mechanism of AcrIIA4 inhibition and confirm the utility of these inhibitors in controlling the myriad functional outputs of dSpyCas9-tethered effector domains (CRISPRi, CRISPRa, and similar tools) [39,40].

The two studies describing Cas9 inhibitors together uncovered seven unrelated Type II anti-CRISPRs (Table 1). In addition, predicted orthologs of some of them were readily identified in additional strains and species [30,41]. These observations suggest that many more Cas9 inhibitors remain to be found, and that Cas9 inhibition is widespread in bacteria.

Accordingly, as in Type I systems, the effects of anti-CRISPRs on Type II CRISPR-Cas evolution and function, host-phage and host-MGE interactions, and horizontal gene transfer (HGT) are likely to be pervasive. Finally, given that Cas9 functions in Type II adaptation (the acquisition of new CRISPR spacers) [47,48] as well as in the interference function of existing spacers [3,14,17,49], it is likely that some or all Cas9 inhibitors restrict Type II CRISPR spacer incorporation, adding an additional layer of broad functional impact to anti-CRISPRs.

Conclusions

Anti-CRISPRs are a fascinating new addition to the CRISPR-Cas world, and many more will surely be discovered. Our appreciation of the uses and impacts of these proteins is only beginning. In particular, the apparent widespread distribution and broad specificity of anti-CRISPRs suggests that they may be neutralizing a significant proportion of observed CRISPR-Cas systems in nature, possibly explaining the continuing occurrence of HGT even when these systems are present [50]. Further investigation will reveal the roles of anti-CRISPRs in driving CRISPR-Cas evolution, potentially helping to account for the tremendous degree of CRISPR-Cas diversity. Studies on anti-CRISPR mechanisms will continue to provide new avenues for elucidating the structures and mechanisms of CRISPR-Cas machineries, and employment of anti-CRISPRs for temporal and tissue-specific control of Cas9 activity holds great promise for improving genome editing approaches. Anti-CRISPRs are one more example of collateral benefits for humans gained from the war between phages and bacteria.

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Highlights

- Viruses and mobile genetic elements evolve countermeasures against CRISPR immunity
- “Anti-CRISPRs” are small proteins that inhibit the CRISPR-Cas machinery
- Type I and Type II CRISPR-Cas systems are inhibited by anti-CRISPRs
- Different anti-CRISPRs can act at distinct stages of target interference

Figure 1.

CRISPR interference machineries that are known to be subject to anti-CRISPR inhibition. CRISPR repeats and spacers are shown as black diamonds and white boxes, respectively, and *cas* genes are given as colored chevrons. **(a)** Type I CRISPR-Cas systems. A representative CRISPR-*cas* locus (subtype I-F) from *Pseudomonas aeruginosa* strain PA14 is depicted; five other subtypes (I-A through I-E, not shown) have also been defined with variations in *cas* gene content. Several Cas proteins, along with processed crRNA, assemble into the Csy complex, which can recognize its crRNA-complementary, PAM-flanked DNA target. R-loop formation enables association of the Cas3 effector (dark blue; in this case, fused to Cas2), which cleaves the non-complementary DNA strand. ATP-driven translocation, combined with additional DNA cleavage events, lead to target destruction. **(b)** Type II CRISPR-Cas systems. A representative CRISPR-Cas locus (subtype II-A) from *Streptococcus pyogenes* strain SF370 is depicted; two other subtypes (II-B and II-C, not shown) have also been defined with variations in *cas* gene content and crRNA biogenesis events. Cas9 (yellow), along with processed crRNA and tracrRNA, form a complex that can recognize the crRNA-complementary, PAM-flanked DNA target. Cas9 then cleaves both DNA strands, and this double-strand break leads to target DNA destruction.

Figure 2.

Anti-CRISPRs can act at distinct stages of Type I and Type II interference pathways. **(a)** Three Type I anti-CRISPRs (AcrF1, AcrF2, and AcrF3) have been characterized mechanistically *in vitro*. AcrF1 and AcrF2 associate with the Csy complex and prevent its association with target DNA; in contrast, AcrF3 (as a homodimer) binds to the Cas2-3 effector, preventing its association with the DNA-bound Csy complex. **(b)** One Type II-C anti-CRISPR (AcrIIC3_{Nme}) and two Type II-A anti-CRISPRs (AcrIIA2_{Lmo} and AcrIIA4_{Lmo}) have each been shown to prevent the functional output of dCas9's target DNA association within cells, indicating that they prevent DNA binding.

Figure 3.

Established strategies for identifying novel categories of anti-CRISPRs. **(a)** Many validated *acr* genes are tightly linked with *aca* genes (thought to encode transcriptional regulators of *acr* expression). Searches for *aca* orthologs can reveal novel, flanking *acr* genes, especially in genomic regions associated with phage-like or MGE-like sequences. Some of these novel *acr* genes appear in the genomes of bacteria that harbor CRISPR-Cas systems of different types (e.g., Type II). **(b)** In many cases, PAM-flanked protospacers are not observed in the same chromosome as matched CRISPR spacers, because CRISPR targeting of the host chromosome can lead to cell death. Expression of an anti-CRISPR (e.g. by an integrated prophage or MGE), however, would be expected to prevent self-targeting-induced cell death. Therefore, bacterial genomes with self-targeting spacers suggests the presence of anti-CRISPR expression. The *acr* gene that prevents autoimmune cell death can then be identified by association with prophage-like or MGE-like sequences, tight *aca* linkage, and other genomic/bioinformatic criteria, as well as by functional tests of CRISPR interference inhibition.

Table 1

Anti-CRISPR proteins reported to date.

Acr Family	Original gene name ¹	Specific example, and source ²	Size (aa)	CRISPR-Cas system(s) inhibited	Citation(s)
AcrE1	JBD5_34	AcrE1 <i>Pae</i> (<i>Pseudomonas aeruginosa</i>)	100	I-E	23
AcrE2	JBD88a_32	AcrE2 <i>Pae</i> (<i>Pseudomonas aeruginosa</i>)	84	I-E	23
AcrE3	DMS3_30	AcrE3 <i>Pae</i> (<i>Pseudomonas aeruginosa</i>)	68	I-E	23
AcrE4	D3112_31	AcrE4 <i>Pae</i> (<i>Pseudomonas aeruginosa</i>)	52	I-E	23
AcrF1	JBD30_35	AcrF1 <i>Pae</i> (<i>Pseudomonas aeruginosa</i>)	78	I-F	22, 24, 27, 28
AcrF2	D3112_30	AcrF2 <i>Pae</i> (<i>Pseudomonas aeruginosa</i>)	90	I-F	22, 24, 27
AcrF3	JBD5_35	AcrF3 <i>Pae</i> (<i>Pseudomonas aeruginosa</i>)	139	I-F	22, 24–26
AcrF4	JBD26_37	AcrF4 <i>Pae</i> (<i>Pseudomonas aeruginosa</i>)	100	I-F	22
AcrF5	JBD5_36	AcrF5 <i>Pae</i> (<i>Pseudomonas aeruginosa</i>)	79	I-F	22
AcrF6		AcrF6 <i>Pae</i> (<i>Pseudomonas aeruginosa</i>)	100	I-E, I-F	29
AcrF7		AcrF7 <i>Pae</i> (<i>Pseudomonas aeruginosa</i>)	67	I-F	29
AcrF8		AcrF8 ZF40 (Pectobacterium phage ZF40)	92	I-F	29
AcrF9		AcrF9 <i>Vpa</i> (<i>Vibrio parahaemolyticus</i>)	68	I-F	29
AcrF10		AcrF10 <i>Sxi</i> (<i>Shewanella xiamenensis</i>)	97	I-F	29
AcrIIA1		AcrIIA1 <i>Lmo</i> (<i>Listeria monocytogenes</i>)	149	II-A	41
AcrIIA2		AcrIIA2 <i>Lmo</i> (<i>Listeria monocytogenes</i>)	123	II-A	41
AcrIIA3		AcrIIA3 <i>Lmo</i> (<i>Listeria monocytogenes</i>)	125	II-A	41
AcrIIA4		AcrIIA4 <i>Lmo</i> (<i>Listeria monocytogenes</i>)	87	II-A	41, 44–46
AcrIIC1		AcrIIC1 <i>Nme</i> (<i>Neisseria meningitidis</i>)	85	II-C	30
AcrIIC2		AcrIIC2 <i>Nme</i> (<i>Neisseria meningitidis</i>)	123	II-C	30
AcrIIC3		AcrIIC3 <i>Nme</i> (<i>Neisseria meningitidis</i>)	116	II-C	30

¹In original publications, the indicated anti-CRISPRs were referred to by their gene names.