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REVIEW ARTICLE

Anti-CRISPRs: The natural inhibitors for CRISPR-Cas systems

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Abstract

CRISPR (clustered regularly interspaced short palindromic repeats)-Cas (CRISPR associated protein) systems serve as the adaptive immune system by which prokaryotes defend themselves against phages. It has also been developed into a series of powerful gene-editing tools. As the natural inhibitors of CRISPR-Cas systems, anti-CRISPRs (Acrs) can be used as the "off-switch" for CRISPR-Cas systems to limit the off-target effects caused by Cas9. Since the discovery of CRISPR-Cas systems, much research has focused on the identification, mechanisms and applications of Acrs. In light of the rapid development and scientific significance of this field, this review summarizes the history and research status of Acrs, and considers future applications.

KEYWORDS
anti-CRISPRs, Cas9, CRISPR-Cas system

1 | INTRODUCTION

CRISPR (clustered regularly interspaced short palindromic repeats)-Cas (CRISPR associated protein) adaptive immune systems are used by certain prokaryotes to resist the invasion of foreign nucleic acids such as phages or plasmids.[1,2] Currently, CRISPR-Cas systems are divided into two classes (1 and 2), six types (class 1: types I, III and IV; class 2: types II, V and VI) and 33 subtypes.[3] Class 1 systems utilize RNA-guided complexes consisting of multiple Cas proteins as the effector proteins to recognize and cleave target DNA, while class 2 systems rely on a single effector-protein (for example, Cas9) with a guided RNA to function. So far, several subtypes of class 2 CRISPR-Cas systems have been identified and developed into powerful gene-editing technologies, including CRISPR-Cas9, Cas12a (Cpf1) and Cas13a (C2c2).[4-7]

In the most widely used CRISPR-Cas9 system, Cas9 nuclease, coupled with a single guide RNA (sgRNA), binds to the target site that is complementary to sgRNA and next to a protospacer adjacent motif (PAM, a short sequence required for binding to the DNA) sequence in the genome, and makes double stranded breaks (DSBs) in the genome. SgRNA is derived from the fusion of CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA).[4] The cell will subsequently initiate DNA repair depending on the availability of repair templates. In the absence of templates, cells will initiate the non-homologous end joining (NHEJ) repair pathway, causing insertion or deletion (indels) mutations at the target site. When an exogenous homologous template is available, the cell will initiate a homology-directed repair (HDR) pathway.[8-10] When Cas9 cleavage activity is completely or partially inactivated, dCas9 (dead Cas9) or nCas9 (Cas9 nickase) will be generated. Based on these Cas9 variants, a number of techniques have been derived by fusing the variants with various functional elements; they include CRISPR-mediated gene activation (CRISPRa), interference with gene expression (CRISPRi), base editing (cytosine base editor, CBE or adenine base editor, ABE), DNA imaging and

epigenetic modification.[11-13] Subsequently the CRISPR-Cas9 system and its derivative gene-editing toolkit have made a promising impact on basic, translational, and clinical research.[7,14,15]

However, the mature CRISPR-Cas9 technologies have as-yet-unresolved off-target problems (cleavage and mutations at unintended sites with high sequence homology to on-target sites), which limits the application of Cas9-mediated technologies.[16,17] There are two general causes of off-target action.[18] The first is the intrinsic specificity of Cas9 protein. Cas9 not only tolerates base pair mismatches in the PAM-distal part of the sgRNA-guiding sequence, it can also tolerate non-optimal PAM sequences albeit with less efficient cleavage activity. Furthermore, Cas9 can cleave off-target sites which have a few extra or missing nucleotides compared with the sequence of the complementary sgRNA. The second cause of off-target action is excessive amounts of Cas9/sgRNA complex, which exacerbate off-target effects. In order to solve this problem, Cas9 protein and sgRNA designs are modified to improve the specificity of target recognition,[19,20] or Cas9/sgRNA abundance and duration are controlled to further decrease off-target effects.[18,21] In recent years, a series of proteins termed anti-CRISPRs (Acrs) have been discovered in bacterial prophage or phages that can inhibit Cas9 cleavage activity.[22,23] Acrs proteins such as AcrIIA4 have been shown to reduce off-target effects by inhibiting Cas9 activity in a timely manner in cells.[24]

Anti-CRISPRs are the natural inhibitors for CRISPR-Cas systems. In the long course of evolution, bacteria and phages have been engaged in an evolutionary arms race.[25] In order to resist infection by phages, bacteria have evolved a number of diverse anti-phage defenses, and one of them is the CRISPR-Cas immune systems.[26] Meanwhile, in order to evade CRISPR-Cas-mediated immunity, phages have evolved the Acr proteins that inhibit the CRISPR-Cas systems.[25] So far, a total of 44 distinct families of Acr genes have been reported (Table 1). Acr proteins are named based on the subtype of the CRISPR-Cas systems they inhibit and the order in which they were discovered.[27] In this review, we summarize the discoveries, inhibition mechanisms and applications of these Acr proteins, and look forward to future developments in their use as research tools and in clinical applications.

2 | THE DISCOVERY OF ACRS

In 2013, the first Acrs were discovered in the phages of *Pseudomonas aeruginosa*. This strain contains the type I-F CRISPR-Cas system and prophage. Researchers found that the prophage sequence in phage-sensitive and -insensitive strains is different. A single unique genetic locus encoding ten distinct protein families was found in the prophage sequence of the phage-insensitive strain, and five of them, AcrIF1-5, could inhibit the type I-F CRISPR-Cas system in *P aeruginosa* (Table 1).[28] Subsequently, another four proteins, AcrIE1-4, were found to inhibit type I-E CRISPR-Cas in *P aeruginosa* (Table 1).[29]

However, these nine Acr proteins originally found in *P aeruginosa* share no homology with the proteins from other bacterial species or phages, and there is no homology among these nine proteins. This makes it difficult to use bioinformatics methods such as Basic Local Alignment Search Tool (BLAST) to find new Acr proteins. However, researchers have found a highly conserved gene downstream of these Acr genes, named Acr-associated gene 1 (*aca1*). *Aca1* encodes a helix-turn-helix (HTH) protein which is a putative transcriptional regulator. Using BLAST, homologous sequences of *aca1* have been retrieved. The genes upstream of these homologous sequences have been cloned into the expression plasmid to interfere with the type I-E and I-F CRISPR-Cas systems of *P aeruginosa*, leading to the discovery of proteins AcrIF6-10 (Table 1), as well as a new Acr-associated gene named *aca2*.[30] It is worth noting that AcrIF6 can function as an inhibitor of both the type I-E and I-F CRISPR-Cas systems.

Three proteins based on the DNA sequence from *aca2*, AcrIIC1-3 (Table 1), were the first inhibitors discovered for the type II-C CRISPR-Cas systems from *Neisseria meningitides*, and these were the first Acr proteins used to regulate Cas9-mediated gene-editing activity in human cells.[22] Another study has found two other type II-C Acrs based on *aca2* (AcrIIC4 and 5, Table 1).[31] This method of using the Acr-associated genes to find novel Acr proteins has been termed "guilt-by-association". Recently, as more bacterial and phage sequence data have been compiled, the "guilt by association" method with further functional assays has led to 12 new Acr proteins being identified, including AcrIC1, AcrIE4-F7 (a chimera), AcrIE5-7, AcrF11-14, and AcrVA1-3 (Table 1).[32] Strikingly, AcrVA1-3 (and AcrVA4-5 discussed below) were the first identified inhibitors for Cas12a (formerly Cpf1).[32,33]

In addition to the "guilt-by-association" concept, another bioinformatic approach based on self-targeting spacers was developed for finding novel Acrs. The idea arose from the phenomenon that some bacterial genomes can be targeted by self CRISPR spacers, whereby the bacterium has to deactivate its own CRISPR-Cas system in order to survive. This phenomenon may imply the presence of the Acr proteins. Subsequently, in this manner, AcrIIA1-4 were discovered (Table 1).[23] Among them, AcrIIA2 and AcrIIA4 have been shown to inhibit the widely used *Streptococcus pyogenes* Cas9 (SpyCas9). In addition, AcrIIA4 has been revealed to significantly limit off-target editing of SpyCas9 in human cells.[24]

Based on this self-targeting bioinformatics analysis, a bioinformatics pipeline named self-targeting spacer search (STSS) has been developed to predict the self-targeting sequence in all available bacterial genomes with the predicted CRISPR arrays. Using STSS combined with a functional screening system called transcription-cell-free translation (TXTL),[34] Kyle E. Watters et al systematically found the inhibitors of Cas12a, namely AcrVA1, AcrVA4, and AcrVA5 (Table 1).[33] Interestingly, AcrVA1 was discovered independently and concurrently by different research groups using two different methods.[32,33]

In addition, A. P. Hynes et al made use of the "phage-first" approach to screen Acr proteins and identified AcrIIA5 and AcrIIA6 in two virulent phages (Table 1).[35,36] AcrIIA5 has proven to be the most broad-spectrum inhibitor of the type II CRISPR-Cas system to date, having been shown to inhibit the type II-A Cas9 proteins (such

TABLE 1 All known anti-CRISPR proteins

Anti-CRISPR	Origin	Number of amino acids	CRISPR-Cas system inhibited	Ref.
AcrIC1	*Moraxella bovoculi* prophage	190	I-C (Pae)	32
AcrID1	*Sulfolobus islandicus* rudivirus 3	104	I-D (Sis)	37
AcrIE1	*Pseudomonas aeruginosa* phage JBD5	100	I-E (Pae)	29,51
AcrIE2	*P aeruginosa* phage JBD88a	84	I-E (Pae)	29
AcrIE3	*P aeruginosa* phage DMS3	68	I-E (Pae)	29
AcrIE4	*P aeruginosa* phage D3112	52	I-E (Pae)	29
AcrIE4-F7	*Pseudomonas citronellolis* prophage	119	I-E/I-F (Pae)	32
AcrIE5	*Pseudomonas otitidis* prophage	65	I-E (Pae)	32
AcrIE6	*P aeruginosa* prophage	79	I-E (Pae)	32
AcrIE7	*P aeruginosa* prophage	106	I-E (Pae)	32
AcrIF1	*P aeruginosa* phage JBD30	78	I-F (Pae, Pec)	28,30,42-45,69
AcrIF2	*P aeruginosa* phage D3112	90	I-F (Pae, Pec)	28,30,42-45
AcrIF3	*P aeruginosa* phage JBD5	139	I-F (Pae)	28,30,42,52,53
AcrIF4	*P aeruginosa* phage JBD26	100	I-F (Pae)	28,30,42
AcrIF5	*P aeruginosa* phage JBD5	79	I-F (Pae)	28,30
AcrIF6	*P aeruginosa* prophage	100	I-E (Pae),/I-F (Pae, Pec)	30
AcrIF7	*P aeruginosa* prophage	67	I-F (Pae, Pec)	30
AcrIF8	*Pectobacterium* phage ZF40	92	I-F (Pae, Pec)	30
AcrIF9	*Vibrio parahaemolyticus* mobile element	68	I-F (Pae, Pec)	30
AcrIF10	*Shewanella xiamenensis* prophage	97	I-F (Pae, Pec)	30,44
AcrIF11	*P aeruginosa* prophage	132	I-F (Pae)	32
AcrIF12	*P aeruginosa* mobile element	124	I-F (Pae)	32
AcrIF13	*Moraxella catarrhalis* prophage	115	I-F (Pae)	32
AcrIF14	*Moraxella* phage Mcat5	124	I-F (Pae)	32
AcrIIA1	*Listeria monocytogenes* prophage J0161a	149	II-A (Lmo)	23,39
AcrIIA2	*L monocytogenes* prophage J0161a	123	II-A (Lmo, Spy)	23,47,48,70
AcrIIA3	*L monocytogenes* prophage SLCC2482	125	II-A (Lmo)	23
AcrIIA4	*L monocytogenes* prophage J0161b	87	II-A (Lmo, Spy)	23,24,47,55-58,71,72
AcrIIA5	*Streptococcus thermophilus* phage D4276	140	II-A (Sth, Spy)	35,36
AcrIIA6	*S thermophilus* phage D1811	183	II-A (Sth)	36
AcrIIA7	Metagenomic libraries from human gut	103	II-A (Spy)	38
AcrIIA8	Metagenomic libraries from human gut	105	II-A (Spy)	38
AcrIIA9	Metagenomic libraries from human gut	141	II-A (Spy)	38
AcrIIA10	Metagenomic libraries from human gut	109	II-A (Spy)	38
AcrIIC1	*Neisseria meningitidis*	85	II-C (Nme, Cje, Geo, Hpa, Smu)	22,31,46,59
AcrIIC2	*N meningitidis* prophage	123	II-C (Nme, Hpa, Smu)	22,31,41
AcrIIC3	*N meningitidis* prophage	116	II-C (Nme, Hpa, Smu)	22,31,41,46
AcrIIC4	*Haemophilus parainfluenzae* prophage	88	II-C (Nme, Hpa, Smu)	31
AcrIIC5	*Simonsiella muelleri* prophage	130	II-C (Nme, Hpa, Smu)	31
AcrVA1	*M bovoculi* prophage	170	V-A (Mb, As, Lb, Fn)	32,33,40,50
AcrVA2	*M bovoculi* prophage	322	V-A (Mb)	32
AcrVA3	*M bovoculi* prophage	168	V-A (Mb)	32
AcrVA4	*M bovoculi* mobile element	234	V-A (Mb, Lb)	33,40
AcrVA5	*M bovoculi* mobile element	92	V-A (Mb, Lb)	33,40,49

Abbreviations: 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*.

as SpyCas9 and SthCas9) in vivo and the type II-C Cas9 proteins in vitro.[34,35] Subsequently, a similar approach was used to identify the first Acr protein inhibiting the type I-D CRISPR-Cas system in the archaeal lytic viruses (AcrID1, Table 1).[37]

Recently, a high-throughput approach was developed to screen for Acr genes from soil, animal, and human metagenomics libraries based on their function of inhibiting SpCas9 rather than on bioinformatics. Four protein families inhibiting Cas9 in vivo and in vitro (AcrIIA7-10, Table 1) have been identified.[38]

3 | ACR MECHANISMS

Among the 44 distinct families of Acr proteins discovered so far (Table 1), mechanisms have been reported for 15 of them, including AcrIE1, AcrIF1-3, AcrIF10, AcrIIA2, AcrIIA4, AcrIIC1-5, AcrVA1, AcrVA4 and AcrVA5. Although structural information has been reported for 14 of these Acr proteins, specific inhibitory mechanisms can be determined for only 11 of them (AcrIE1, AcrIF1-3, AcrIF10, AcrIIA2, AcrIIA4, AcrIIC1-3, and AcrVA5). The structural information reported for another three Acr proteins (AcrID1, AcrIIA1, AcrIIA6) did not clearly illuminate their mechanisms.[36,37,39] In addition, the inhibitory mechanisms of AcrIIC4, AcrIIC5, AcrVA1, and AcrVA4 were determined by biochemical experiments.[31,40] The known mechanisms of these 15 Acr proteins can be roughly divided into three types: crRNA loading interference, DNA binding blockage and DNA cleavage prevention. This corresponds to the three steps of CRISPR-Cas-mediated immunity through direct interference with foreign DNA (Figure 1).

3.1 | crRNA loading interference

According to a recent study,AcrIIC2 has been identified as interfering with crRNA- and DNA-loading steps through binding to a Cas9 BH motif.[41] AcrIIC2 is the first reported Acr protein interfering with crRNA loading, which prevents the correct assembly of the crRNA-Cas9 complex, resulting in the blockade of Cas9 activity.

3.2 | DNA binding blockage

In addition to AcrIIC2, 11 other Acr proteins can block the target DNA binding, but the mechanisms by which they block DNA binding are completely different. Structural information has shown that AcrIF1, AcrIF2, and AcrIF10 act on different subunits of the Cascade effector complex of the type I-F CRISPR-Cas system to prevent DNA binding to the complex.[42-45] Biochemical and structural data suggested that AcrIIC3 promotes dimerization of Cas9 and prevents DNA binding.[41,46] The structure of the Cas9-sgRNA-AcrIIA4 complex, revealed by 3.9 Å resolution cryo-electron microscopy, indicates that AcrIIA4 binds to the PAM-interacting domain of Cas9, thus preventing the target DNA binding.[24,47] What is more, AcrIIA4 binds only to assembled Cas9-sgRNA complexes, not to Cas9 protein alone or to preformed Cas9-sgRNA-DNA complexes.[23,24] Combining electrophoretic mobility shift assays (EMASs), fluorescence polarization assays and image assays, J. Lee et al showed that both AcrIIC4 and AcrIIC5 prevent NmeCas9 from binding to DNA while having no effect on sgRNA loading.[31] Recently, two structural studies revealed that AcrIIA2 acts as a DNA mimic, blocking the PAM recognition residues and

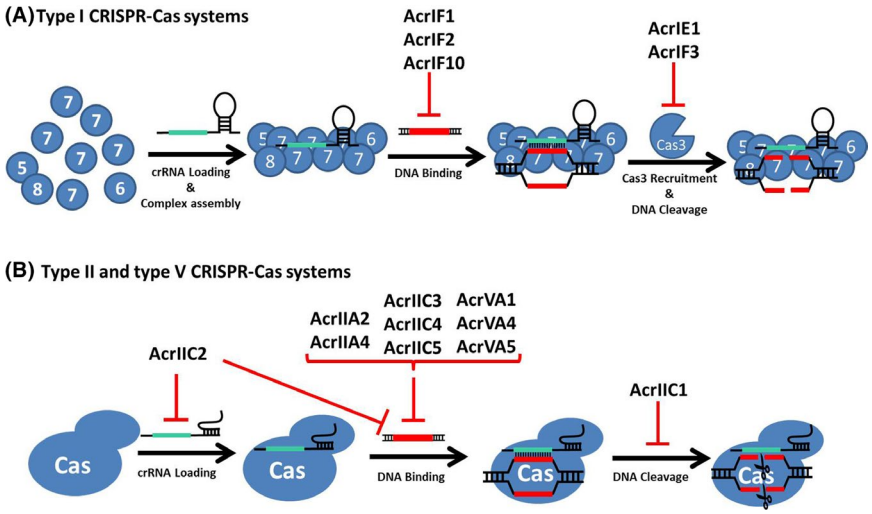

FIGURE 1 Schematics of anti-CRISPR protein interfere with different stages of type I, type II and type V CRISPR-Cas systems. A, In type I CRISPR-Cas systems, the nine subunits of the Cascade come together with the CRISPR RNA (crRNA) to form the surveillance complex, which uses the spacer sequence (cyan) to search for target DNA (red). Then the Cas3 nuclease is recruited to the complex and cleaves the target DNA. AcrIF1, AcrIF2, and AcrIF10 can block the target DNA binding. AcrIE1 and AcrIF3 prevent Cas3 recruitment and thereby prevent DNA cleavage. B, In type II and type V CRISPR-Cas systems, the crRNA is loaded onto Cas protein (Cas9 or Cas12a) to form a ribonucleoprotein complex, which binds to target DNA and then cleaves it. AcrIIC2 is shown to interfere with crRNA- and DNA-loading. AcrIIA2, AcrIIA4, AcrIIC3, AcrIIC4, AcrIIC5, AcrVA1, AcrVA4 and AcrVA5 are known to block the target DNA binding. AcrIIC1 prevents the target DNA cleavage

subsequently preventing dsDNA recognition and binding, which is similar to the actions of AcrIIA4.[48]

In addition, two recent studies showed that AcrVA1, AcrVA4, and AcrVA5 robustly block Cas12a binding to DNA via distinct mechanisms.[40,49] AcrVA1 triggers endoribonuclease activity to truncate the Cas12a bound crRNA and permanently inactivates the Cas12a surveillance complex. AcrVA4 blocks dsDNA binding by driving dimerization of Cas12a-crRNA complexes, similar to the action of AcrIIC3.[40,46] Structural and biochemical data revealed that AcrVA5 can block target DNA binding through acetylating the lysine residue of the PAM recognition region of *Moraxella bovoculi* (Mb) Cas12a.[49] These two studies revealed a previously unobserved mechanism whereby AcrVA1 and AcrVA5 use enzymatic activities rather than physical barriers to shut down the Cas12a endonuclease activity.[50]

3.3 | DNA cleavage prevention

Three Acrs can inhibit CRISPR-Cas systems by preventing target DNA cleavage. X-ray crystallography showed that AcrE1 binds to the CRISPR-associated helicase/nuclease Cas3.[51] Both biochemical assay and structural analysis revealed that AcrIF3 binds directly to Cas3 as a dimer and prevents the recruitment of Cas3 to the Cascade complex.[42,52,53] Detailed biochemical and structural characterization demonstrated that AcrIIC1 directly binds to the active site of the HNH endonuclease domain in Cas9, which prevents DNA from cleaving and transforms Cas9 into an inactive but DNA-bound state.[46]

3.4 | Function and applications

The first application of Acrs was to regulate Cas9- or Cpf1-mediated gene editing in human cell lines. Given that unintended DNA modification and cleavage by off-target Cas nuclease activity is permanent, high specificity is particularly important in Cas9- or Cpf1-mediated gene therapy.[7] Many type II-A, type II-C, and type V-A Acrs can inhibit Cas9- or Cpf1-based genome editing in human cell lines.[22-24,31,33,36] Notably, a research group has shown that timed addition of AcrIIA4 can significantly reduce off-target editing at some tested off-target sites in human cells,[24] which indicates a potential clinical application in the future.

Anti-CRISPRs can be a robust "off-switch" for CRISPR-Cas systems. For example, type II-A or type II-C Acr proteins can inhibit the Cas9-based gene drive, which has been developed for eradicating disease vectors such as mosquitos over a long timeframe.[54] Acrs can avoid the unpredictable ecological consequences caused by gene drives based on Cas9. A recent study has shown that AcrIIA2 and AcrIIA4 proteins can inhibit active gene drive systems in budding yeast.[55]

Some of the type II Acr proteins can also inactivate dCas9-based genome editing technologies by blocking dCas9 binding to the target DNA. Several studies have revealed that AcrIIA4 can significantly inhibit gene regulation by CRISPRi, CRISPRa and targeted DNA demethylation in human cells.[56,57] Moreover, optogenetic controlled AcrIIA4 and inducible AcrIIA4 can modulate Cas9-mediated genome or epigenome editing.[57,58]

Interestingly, a recent study reported a centrifugal microfluidic platform to detect both Cas9 protein levels and nuclease activity.[59] In this platform, AcrIIC1 was initially used as a capture reagent to detect Cas9 from several species. Thus Acrs can be potentially used to detect accidental exposure, malicious use, and undesirable persistence of Cas9.

Another potential use of Acrs is phage therapy. With the emergence of many drug-resistant bacteria, phage therapy is considered as an alternative to antibiotics. However, some pathogenic bacteria such as *P aeruginosa* and *Clostridioides* harbor CRISPR-Cas systems, which prevent phage propagation and lysis in host bacterial cells.[60-62] Engineered phages that contain Acrs could help phage therapy overcome this limitation.[63,64]

4 | OUTLOOK

Although it is a novel research field, the study of Acrs is not limited to their discovery, mechanisms and applications. The origin of Acrs and the potential evolutionary consequences for CRISPR-Cas systems or horizontal gene transfer are research hotspots.[25,64-67] Meanwhile, it is worth investigating whether the target bacteria have so-called anti-Acrs strategies to protect themselves from phages carrying Acrs.[64,66,67] Like the discovery of restriction enzymes and CRISPR-Cas9 technology, the study of Acrs as the natural inhibitors for CRISPR-Cas systems will contribute to the phage-host interaction field, which may lead to the emergence of novel biotechnologies.

There are also remaining questions about the discovery and mechanism of Acrs. Although Acrs have been identified for partial subtypes of type I, type II, and type V CRISPR-Cas systems, most CRISPR-Cas inhibitors are unknown. The subtype II-B CRISPR-Cas9 system, such as FnCas9 (*Francisella novicida* Cas9), and type VI CRISPR-Cas13a (formerly C2c2, which is a mature RNA editing tool) do not have known inhibitors.[6,68] In addition, the inhibitory mechanisms of many Acrs, such as AcrIIA5-10, AcrVA2-3, etc, have not been described. Determining these mechanisms will help researchers develop versatile genome engineering modulators or specific applications. Meanwhile, elucidating the diversity of Acr mechanisms will further increase our understanding of how phages and bacteria compete in the evolutionary battle for their survival.

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CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTIONS

FZ conceived and wrote the original draft of the manuscript. GXS and YT revised the manuscript. All authors critically read and contributed to the manuscript, approving its final version.

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