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Anti-CRISPR: A defense strategy of bacteriophages against bacteria

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Abstract
Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas system is a sequence-specific adaptive immune strategy, widespread across prokaryotic systems conferring innate immunity against various bacteriophages and other MGEs like plasmids, genomic islands, integrative and conjugative elements. Even with the presence of such a sophisticated mechanism, the bacteria haven't been always completely victorious against phages. This is due to Anti-CRISPR proteins produced by bacteriophages and other MGEs. Since their discovery in 2013, more than 60 Acr families have been identified to date and many more are yet to be known. Studies revealed diverse mechanisms adopted by Acrs through which they mediate their control over CRISPR defense system. With the development in this area, Acrs can be harnessed as potential control strategies for CRISPR-Cas technology. In this review, we focus on the discovery of various Anti-CRISPR proteins, their mechanism of action through which they can counteract the bacterial CRISPR-Cas system, and the potential applications of Acrs in gene editing and gene therapy technologies.

Keywords: Anti-CRISPR, bacteriophage, Cas protein, CRISPR, genome editing

Introduction
Phages and bacteria have been at war for millions of years where phages control the number and composition of the bacterial population. To combat this constant threat from phages, bacteria have evolved very diverse defense strategies that put checkpoints at various stages of phage's life cycle. This includes phage attachment blockade, inhibiting DNA entry, development of restriction-modification systems, abortive infection systems (Abi), and interfering with phage assembly [1]. In addition to the above strategies bacteria have evolved a sequence-specific adaptive immune strategy called CRISPR Cas [2]. CRISPR arrays are the storehouses of data regarding the phages that have previously infected the bacterial cell. Cas proteins together with the CRISPR arrays constitute this RNA-guided nuclease complex. The host cell differentiates the self DNA and that of the invading foreign mobile genetic elements with the help of a short sequence called Protospacer Adjacent Moiety (PAM) [3].
As part of adaptive immunity, all the systems act through three main stages: adaptation or spacer acquisition, expression or biogenesis, and interference phase. In the first stage, the Cas1–Cas2 complex recognizes PAM and excising small portions of the target DNA called protospacers which are then integrated into CRISPR locus as spacer sequences. Other accessory factors such as Cas4, Cas1, Csn2, and reverse transcriptase (RT) can also be involved in the acquisition phase. In the next stage, the CRISPR locus is transcribed into a single pre-crRNA which is then processed into mature CRISPR RNAs (crRNAs) [4]. Each crRNA contains parts of the flanking repeats and a spacer sequence. At the interference stage, crRNA is recruited along with the Cas proteins to form ribonucleoprotein complexes, which go on surveying the cell for any matches with crRNA spacer sequences. If found, the nuclease activity is initiated either by the recruitment of new proteins or activation within the complex itself based upon the class of CRISPR Cas system.
The CRISPR-Cas systems are grouped into two classes, six types and over 30 subtypes [5, 6, 7]. Class 1 CRISPR Cas system includes I, III, and IV types and uses multi-subunit Cas effector molecules to form Cascade complex. Whereas in Class 2 systems (II, V, and VI types) the target recognition, binding, and cleavage functions are carried out by single effector proteins.
Due to this highly diverse and efficient mechanism, the CRISPR Cas system may confer protection to bacteria not only from the phages but also from other Mobile Genetic Elements (MGEs) like plasmids, genomic islands, integrative and conjugative elements [8].

Therefore it is possible to produce a robust and safer bacterial population with increased phage resistance and targeted interference with the distribution of the antibiotic resistance marker carrying plasmids [9].

Phages strike back: Anti-CRISPR Discovery

Owing to the bacterial defensive measures, phages have also strategized certain ways to evade this prokaryotic CRISPR-Cas system. One such way is through the acquisition of point mutations [10]. As discussed earlier, a crRNA guided match between spacer in CRISPR locus and protospacer in MGEs is required for the nuclease activity but mutations in the conserved PAM region evade this highly specific defense even in the presence of a perfect spacer-protospacer match [11]. At the same time mutations in the protospacers which confer mismatches inhibit CRISPR/Cas activity even though the PAM region is intact [11]. But hosts tend to restore their immunity by rapidly acquiring new spacers through positive feedback called "Priming". By this robust process, even outdated spacers with many mismatches will be able to stimulate a rapid response giving the bacteria an advantage in an arms race with their opponents [12]. To evade the adaptive nature of this immune system, phage needs a different strategy where it can inactivate CRISPR-Cas system in a sequence-independent manner.

In 2013 the counteractive Anti-CRISPR proteins were found in phages that infect *Pseudomonas aeruginosa* [13]. Single locus coding for ten different proteins was identified through sequence analysis, out of which only five genes (acrF1-acrF5) were found with the ability to counteract the type I-F system in *Psuedomonas aeruginosa* through AcrIF1-5 proteins. But more than half of the genes in this operon didn't show any anti-CRISPR activity against type I-F systems [13]. Follow up studies revealed the presence of the second set of proteins comprising four distinct families (AcrIE1-4) acting against the type I-E system [14]. The loci of the genes coding for these two types of anti-CRISPR proteins showed a lot of similarity suggesting that these two groups worked together.

Search for new Anti-CRISPR proteins

Guilt by approach

It was found later that the phages which encoded for the Acrs have also encoded for Aca1 (Anti-CRISPR associated 1 and found downstream to the then known anti-CRISPR gene loci and more interestingly absent in those phages lacking anti-CRISPR activity. It was observed that this Aca contains a helix-turn-helix motif which is commonly found in transcriptional regulators speculating the fact that Aca proteins may regulate the expression of Acr operon. By adopting the 'guilt by association' bioinformatics approach, a BLAST search was conducted using Aca 1 to find out the proteins encoded by genes upstream of Aca1 homologs [15]. Proteins were identified on the basis of their gene location that they should be located on the same strand as that of the aca 1 and should be of less than 200 amino acid residues as all the previously found anti-CRISPRs were in the range of 50-139 residues. These BLAST queries revealed homologous in many members of Proteobacteria. Further cloning of these homologous into expression plasmids followed by bacteriophage plaque assay revealed another new set of anti CRISPR protein families- AcrIF6-10 inactivating type I-F system. It was also found that AcrIF6 (pae) inactivated both type I-F and type I-E systems which were totally unexpected. After the initial discovery of AcrIF6 and its homologous, a gene coding for HTH protein was found downstream to the active anti-CRISPR AcrIF6 (*Oceanimonas smirnovii*) encoding gene. Assuming that this helix-turn-helix protein is also associated with Acrs, genes coding for anti-CRISPRs were found upstream to the genes coding for homologous of HTH protein leading to the discovery of Aca 2, a new family in Aca proteins [15].

Hypothesizing that the anti-CRIPSRs inhibiting type II systems would also exist, the same bioinformatic approach which was previously employed was used to search for Cas 9 inhibitors. BLAST searches with Aca 2, a candidate anti-CRISPR gene coding for 91 residues hypothetical protein in *Brackiella oedipodis* was identified upstream to aca 2 gene. On finding that the homologous of this protein existed in the species possessing type II-C CRISPR-Cas system and considering the fact *N. meningitides* strain 8013 harbors a well-established type II-C system, NmeCas9 was employed to trace the genes encoding for Cas 9 inhibitors. This led to the discovery of three anti CRISPR protein families (AcrIIC1-3) [16]. After the initial discovery of acrIIC1, it was observed that all its homologous except acrIIC1 (Boe) were not found adjacent to aca 1 and aca 2 genes but upstream to a variety of genes coding for HTH proteins. This fact led to the discovery of a new member of the Aca family- Aca 3 protein [16], whose BLAST queries led to the discovery of AcrIIC2Nme and AcrIIC3Nme proteins. In 2018, two other novel anti-CRISPR families (AcrIIC4, 5) were reported in strains of *Haemophilus parainfluenzae* and *Simonsiella muelleri* [17].

Employing the same 'guilt by association', BLAST queries for the search of aca1 homologous in *Pseudomonas* genomes led to the discovery of seven new gene families upstream of aca1 [24]. They were acrIE5-7, acrIF11, two genes with no inhibitory activity (orf1 and orf2), and a chimera, acrIE4-F7 which was able to inhibit both type IE and IF systems acrIF11 was found associated with genes coding for DNA binding motifs which were characterized as aca4-7 [24]. In search of other acr genes, aca4 was used to identify a new *Pseudomonas* Anti-CRISPR, acrIF12 [24]. Two more types IF Acrs (AcrIF13, 14) and Acrs inhibiting type I-C and V-A systems (AcrIC1, AcrVA1–3) were found while investigating for genes adjacent to acrIF11 in *Moraxella* strains [24]. It was also found that the acrVA1 gene potently inhibited Cas12a in both human and bacterial cells providing a new tool for the regulation of Cas12a [24].

Self –targeting spacers:

In 2017, a bioinformatics tool based on 'self-targeting' was employed trace type II-A CRISPR Cas 9 inhibitors [19]. According to this bacteria which contain spacers targeting self-genes, require inactivation of CRISPR-Cas to avoid self-destruction [13, 18]. This led to the discovery of four types of II-A CRISPR Cas 9 inhibitors in *Listeria monocytogenes* namely AcrIIA1, AcrIIA2, AcrIIA3, and AcrIIA4 [19]. Among these proteins, it was found that AcrIIA2 and AcrIIA4 were able to block SpyCas9 activity in bacterial and human cells [19].

Phage-first approach

There were other means employed for the discovery of new anti-CRISPRs like the identification of self-targeting spacers and phage-oriented approaches [10]. In a phage-oriented approach, phages were screened for their ability to escape CRISPR mediated immune response [20]. During this process, *Streptococcus thermophilus* DGCC7854 strain organisms were infected with a set of five virulent phages. Out of these

five phages, host cells were able to develop immunity against four but not against phage D4276. Individual genes from the resistant phage D4276 are systematically cloned onto plasmids and expressed in *Streptococcus thermophilus* followed by plating with CRISPR sensitive phage to check for phage titer which led to the discovery of acrII5 gene [20]. These Acr homologous were detected in virulent phage genomes making AcrIIA5 the first anti-CRISPR to be discovered in the virulent phage [20]. In 2018, another protein named AcrIIA6 was found in *S. thermophilus* phage D1811 [21]. 38% of the analyzed virulent *S. thermophilus* phages possessed either AcrIIA5 or AcrIIA6, whereas all temperate phages had one of these two Acrs [21], confirming that virulent phages can also carry Acr genes and these Acrs are closely related between temperate and virulent phages. In the same study out of all the Acrs tested AcrIIA5 showed the broadest activity and in a concurrent *in vitro* study, AcrIIA5 was able to inhibit type IIC systems of *Neisseria meningitides* and *Campylobacter jejuni* [22].

In the search for archaeal Acrs, a similar approach was employed in lytic rudivirus SIRV2 infecting crenarchaeon *Sulfolobus islandicus* LAL14/1 (23) possessing I-D, I-A, and III-B systems. LAL14/1 has 5 repeating CRISPR arrays with 13 spacers targeting SIRV2 with few mismatches, but still, the phage was able to infect its host suggesting the existence of anti-CRISPR proteins. A mutant of SIRV2 (SIRV2M) was characterized by a 4kb deletion with no ability to infect LAL14/1. But infectivity of SIRV2M was restored on cloning with SIRV3 genes (each gene cloned individually) which were found in the deleted fragment. The gene which was able to restore the infectivity was identified and it was shown that this gene coded for Acr ID1 which directly interacts with Cas10d subunit [23]. Acr ID1 was the first Acr discovered in archaeal phages and it is also the first one of its kind acting against type I-D CRISPR-Cas systems [23].

Self-Targeting Spacer Searcher

As a next step in the discovery of Acrs, Self-Targeting Spacer Searcher (STSS) was adopted as a bioinformatic pipeline to search for self-targeting examples in the NCBI prokaryotic database [25]. It employed CRISPR Recognition Tool (CRT) to predict all possible self-targeting spacers in bacterial genomes containing CRISPR arrays [26]. Four strains of *M. bovoculi* were identified to contain self-targeting spacers for type-V system and a cell-free transcription-translation (TXTL) system was employed [22] to confirm the presence of Acr genes in their genomes. As a result AcrVA1, AcrVA4, and AcrVA5 proteins were found to inhibit Cas12a activity in TXTL assay [25].

Identification of Acrs from Metagenomic libraries

In the quest for further Acrs, researchers developed a synthetic circuit to screen for Acrs against type IIA systems in metagenomic libraries [27]. By cloning SpyCas9 with guide RNA that targets the antibiotic resistance gene, a genetic circuit was designed to rapidly select metagenomic libraries containing Acrs using simple antibiotic selection. As a result of four Cas9 antagonists, AcrIIA7–10 were identified [27]. A similar search strategy was used to recover AcrIIA11 from a *Lachnospiraceae* phage [28]. AcrIIA11 was able to inhibit spyCas9 of both human and bacterial cells and its homologous are distributed across diverse bacteria. Its interaction with conserved residues of Cas9 can be implied from the broad spectrum of inhibition. It was said that AcrIIA11 binds both spyCas9 and dsDNA and inhibits DNA cleavage [28]. The exact mechanism is yet to be elucidated but AcrIIA11's is different from previously characterized typeIIA Acrs [19, 20, 21, 27].

Other Anti-CRIPSR proteins

AcrIIA1 exhibits a broad spectrum inhibitory effect on Type II-A and II-C Cas9s including a highly diverged *Listeria* Cas9 [19, 25, 29]. But during lytic cycle, AcrIIA1 alone isn't enough for Cas9 inactivation and requires additional Acrs to inhibit Cas9-DNA binding [29]. Probing into this, researchers identified acrIIA12 which is found in every Acr locus coding for acrIIA1. This new Acr was able to inhibit LmoCas9 but not SpyCas9 [29]. By employing STTS and Guilt-By-Association approaches to screen for SauCas9 inhibitors, researchers identified three new bifunctional SauCas9 inhibitors: AcrIIA13–AcrIIA15 [30]. These new Acrs share a common N- terminal region and different C-terminal regions which are speculated to be responsible for inhibition of SauCas9 mediated target DNA cleavage via distinct mechanisms [30]. Till recent times the Anti-CRISPR genes have been discovered in the genomes of phages or prophages. In 2020, Mahendra *et al*., used *Listeria* acrIIA1 gene as a marker to identify acr loci (four distinct inhibitors acr IIA16-19) on the various MEGs of Firmicutes [31]. It was understood that these Acrs protect plasmid DNA during conjugation and phage DNA during infection. *in vitro* studies suggested that AcrIIA16-19 inactivate Cas9 mediated cleavage of foreign DNA and *in vitro*, they might modulate the expression, stability, and loading of sgRNA [31].

Recently a III-B system inhibitor called AcrIIIB1 was identified in *Sulfolobus* virus SIRV2 [32]. AcrIIIB1 was found to inhibit type-IIIB associated RNase called Csx1. In the search for other archaeal Acrs, a new family of Acrs (AcrIII-1) that rapidly degrade cyclic tetra-adenylate (cA4) was identified [33]. As it targets cA4 signaling molecules rather than specific CRISPR effector proteins; this family of Acrs has a broad host range. This viral ring nuclease is widely distributed in archaeal and bacterial viruses and also in provirus [33]. Smargon *et al*. identified two type VI-B CRISPR systems that lack Cas1 and Cas2 and contain Cas13b [34]. Cas13b is a RNA guided RNase with Csx27 repressing and Csx28 enhancing its activity. Csx27, although appears to be a Cas protein represses Cas13b-mediated RNA interference [34, 35]. Recently two new Anti-CRIPSRs: AcrIIA20 and AcrIIA21 were identified using a combination of STTS and a machine learning-based method called AcRanker [61]. AcrIIA20 contains only 64 amino acids making the smallest known Acr to date. AcrIIA20 inhibits SinCas9 with high potency and SauCas9 with low potency whereas AcrIIA21 has a broader inhibition range. Researchers speculate that due to its high negative charge, AcrIIA20 may inhibit DNA cleavage by a mechanism similar to that of PAM mimicry; whereas nucleic acid binding might be the potential mechanism of inhibition employed by AcrIIA21 [61]. A webserver implementation for AcRanker is available at http://acranker.pythonanywhere.com/

By using an integrated approach with STTS and TXTL systems to screen and characterize Cas13a inhibitors, researchers identified a series of acrV1-7 genes [62]. These Acrs can interact with Cas13 or the Cas13-crRNA binary complex and inhibit Cas13a based RNA targeting in both human and bacterial cells.

A new web server called AcrFinder

(http://bcb.unl.edu/AcrFinder) was developed recently to screen the genomic data for potential Acr candidates. The new software package (https://github.com/HaidYi/acrfinder) takes both nucleotide and protein sequences as inputs, while the previous standalone software package, AcRanker allows only protein files as inputs. This system contains Acr-Aca database and employs various bioinformatics tools like homology-based, self-targeting, GBA based approaches integrated in a single software [60].

Nomenclature and database for Anti-CRISPR Proteins

A system for nomenclature of Acr proteins and genes was introduced in 2015 [16]. It is established as: the type of CRISPR Cas system inhibited, followed by a numerical value representing the protein family given in the order of its discovery and then a subscript representing the source of that protein [38]. For example: an Acr inhibiting type IF system and in 9th position the order of its discovery is represented as AcrIF9. Bondy-Denomy *et al*. establish a database for the registration and tracking of anti-CRISPR names [38]. The database can be found in : https://tinyurl.com/anti-CRISPR. There is another anti-CRISPR database described by Dong *et al* in 2018 [39]. Some of the currently available bioinformatic tools for Acr research include CRISPRminer [64], Self-targeting Spacer Searcher [25], AcrCatalog [63], AcrFinder [60] and AcRanker [61].

Anti-CRISPR mechanisms

Class I Acrs

Class I Acr proteins discovered till now inhibit type I CRISPR-Cas system. Some of their mechanisms are still unknown while some Acr proteins block DNA cleavage by interacting with Cas 3 nuclease, the most common way is by interacting with the cascade complex and preventing DNA binding [40].

AcrIF1 uses three different binding modes for inhibiting the cascade complex in which two molecules each binding to Cas7f.3-Cas7f.4 and Cas7f.5- Cas7f.6 interfaces respectively by interacting with the basic residues on Cas7f creates a steric hindrance for crRNA to access the target DNA. Another binding mode adapted by AcrIF1 is binding to Cas7f.6, which is in close proximity to the region crucial for DNA binding [13, 41, 42].

AcrIF2 acts by DNA mimicry as the negatively charged surface gives it a DNA duplex resemblance by exhibiting a pseudo helical distribution. Thereby interacts with basic residues on type I-F Csy complex and sterically hinders the access of dsDNA to cascade complex by pushing the Cas8F hook away from Cas7.6f and bringing out conformational change [13, 41]. In the same way, AcrIF10 acts by DNA mimicry and interacts with the groove formed by Cas7f.6 and the Cas8f hook which normally accommodates for target dsDNA binding thereby locking Cas8f in a DNA binding fashion [15, 43]. The archaeal Acr protein, AcrID1 directly interacts with Cas10d protein which is the large subunit of effector complex thereby blocking the DNA binding stage. The high density of negative charge on this protein suggests that this Acr may also act as a DNA mimic [23, 40].

X ray crystallography and cryo EM studies revealed that AcrIF3 dimer directly form a complex with Cas3 nuclease with extensive hydrophobic interactions and hydrogen bonds thereby preventing the crRNA interference and target DNA cleavage by maintaining Cas3 in an inactive ADP bound form [13, 40, 44, 45]. In addition, it was revealed that AcrF3 blocks Cas3 activity by directly binding with Cas1-2/3 complex, explaining its ability to block the spacer acquisition too [46]. AcrIE1 also binds with ATP dependant Cas3 to inhibit target DNA cleavage but the exact mechanism is not known [14, 47].

Class II Acrs

AcrIIA4 inhibits Cas9 nuclease activity by multiple mechanisms like : a) interaction with RuvC active site thereby blocking nuclease activity, b) Mimics PAM region and binds to PAM interacting domain (PID) of Cas9 thereby preventing DNA binding, c) impeding the conformation change of HNH domain, d) inhibit target dsDNA unwinding by interacting with phosphate lock loop [19, 48, 49, 50, 51]. Similarly AcrIIA2 associates with WED domain, PAM interacting domain, HNH, and REC2 domains and overlaps with AcrIIA4 while binding with PID [40, 52, 53].

AcrIIC2 due to its high negative charge, interacts with arginine-rich bridge helix thereby hinders sgRNA loading to Cas9 [54]. AcrIIC1 interacts with highly conserved catalytic residues present on HNH domain of NmeCas9 thereby creating a catalytically dead Cas9 [55]. Whereas AcrIIC3 binds to the surface opposite to the active site of HNH domain which may allow PAM detection but inhibits R loop formation. In addition to this AcrIIC3 interacts with REC lobe which causes AcrIIC3-Cas9 dimerization which prevents the loading stage. AcrIIC3 specifically binds only with NmeCas9 [54, 55]. Both AcrIIC4 and AcrIIC5 bind to Cas9 and inhibit DNA binding stage but the exact mechanism isn't clearly understood [17].

AcrIIA6 recognizing sites and PAM are structurally close and thereby acts as an allosteric inhibitor of Cas9 and induces its dimerization [59]. AcrIIA11 was the 1st ever reported Acr that can bind with both dsDNA and Cas protein. It uses a distinct mechanism to bind with the conserved residues on Cas9 inhibiting DNA cleavage but not the target recognition [28]. AcrIIA13-15 share a common conserved N terminal region and distinct C terminal regions speculated to be responsible for DNA cleavage inhibition [30]. AcrIIA16-19 interacts with Cas9 via a distinct mechanism thereby preventing Cas9 mediated DNA cleavage; studies show that these Acrs are able to modulate sgRNA expression, loading, and stability [31].

A cellular enzyme named viral ring nuclease (Acr III-1) specifically binds with cA4 and rapidly cleaves the signaling molecule thereby neutralizing type III CRISPR defense system [33]. Generally, the IIIB1 systems require an additional RNase called Csx1 while targeting middle or late genes. AcrIIIB1 inhibits Cmr-α and Cmr-ɣ complexes which are required for cyclic oligoadenylates synthesis thereby inhibiting Csx1 signaling pathway [32]. It was also found that AcrIIIB1 doesn't show any effect on early gene targeting or Cas10 DNase activity. Csx27, although appears to be a Cas protein represses Cas13b-mediated RNA interference [34].

AcrVA5 acts as an acetyltransferase and acetylates the lys residues on Cas12a that are required for PAM recognition thereby preventing binding of dsDNA substrates to Cas [57]. AcrVA4 binds with residues on REC domain which are involved in pre-crRNA processing and crRNA binding, maybe by mimicking pre-crRNA, bringing out the conformational changes which further blocks the target DNA binding [56, 65]. AcrVA1 due to high negative charge on its surface, mimics the PAM of target DNA and binds with Cas12a and subsequent cleavage of crRNA by its RNase activity [56, 58].

Applications of Anti-CRISPR proteins

Due to the growing importance of CRISPR-Cas technology, the application areas of Anti-CRIPSRs are also tremendous. Importantly Acrs can be used to regulated Cas mediated activities to minimize its off-target effects without disturbing the on target ones [71]. Acrs have been applied for the regulation of CRISPR interference and CRISPR- activation in both bacterial and mammalian cells [66]. They can also be used as section markers for engineering viral genomes acrD1 was used as a selection marker for knocking out the selected genes from SIRV2 [69]. CRISPR-Cas systems have been reported in conferring bacteria with increased virulence, aggravating the bacterial pathogenicity. Acrs can be used to check and control these.

The importance of phage strategy has been increasing due to the alarming threats from anti-bacterial resistance and the CRISPR mediated immune strategy has been a great hurdle in this field. But this problem be overcome by equipping therapeutic phages with Acrs which protect the phages from destruction while they mediate them carry out their anti-bacterial effects [67]. Engineered optogenetic Acr variants, when co-expressed with CRISPR effector molecules were able to mediate both genome and epigenome editing [68].

Acr proteins can be applied to modulate or inhibit the drive strength. Recently AcrIIA2 and AcrIIA4 mediated inhibition of gene drives has been demonstrated in yeast model system [70]. miRNA regulated Acr switches were used for cell type specific activation or inactivation of Cas9 [72]. This can greatly reduce the effect of Cas nuclease activity on unintended cells of a given group of cells or organisms. *In vivo* effects of this strategy was demonstrated by Lee *et al*., in adult mice by restricting Nme2Cas9 activity to only liver cells while sparing the heart cells [73]. Acrs were also used in production of helper dependant adeno virus vector where in AcrIIA2 and AcrIIA4 were used to inhibit viral self-cleavage by SpyCas9 [74].

Table 1: Anti-CRISPR protein families

Name	type inhibited	Stage inhibited	Originating species	Ref
AcrIC1	I-C	Unknown	*Moraxella bovoculi* prophage	24
AcrID1	I-D	DNAbinding	*Sulfolobus islandicus rudivirus* 3	23
AcrIE1	I-E	DNA cleavage	*Pseudomonas aeruginosa phage* JBD5	14
AcrIE2	I-E	Unknown	*P. aeruginosa phage* JBD88a	14
AcrIE3	I-E	Unknown	*P. aeruginosa phage* DMS3	14
AcrIE4	I-E	Unknown	*P. aeruginosa phage* D3112	14
AcrIE4-F7	I-E/I-F	Unknown	*Pseudomonas citronellolis* prophage	24
AcrIE5	I-E	Unknown	*Pseudomonas otitidis* prophage	24
AcrIE6	I-E	Unknown	*P. aeruginosa* prophage	24
AcrIE7	I-E	Unknown	*P. aeruginosa* prophage	24
AcrIF1	I-F	DNAbinding	*P. aeruginosa* phage JBD30	13
AcrIF2	I-F	DNAbinding	*P. aeruginosa* phage D3112	13
AcrIF3	I-F	DNA cleavage	*P. aeruginosa* phage JBD5	13
AcrIF4	I-F	DNAbinding	*P. aeruginosa* phage JBD26	13
AcrIF5	I-F	Unknown	*P. aeruginosa* phage JBD5	13
AcrIF6	I-F	Unknown	*P. aeruginosa* prophage	15
AcrIF7	I-F	Unknown	*P. aeruginosa* prophage	15
AcrIF8	I-F	Unknown	*Pectobacterium* phage ZF40	15
AcrIF9	I-F	Unknown	*Vibrio parahaemolyticus* mobile element	15
AcrIF10	I-F	DNAbinding	*Shewanella xiamenensis* prophage	15
AcrIF11	I-F	Unknown	*P. aeruginosa* prophage	24
AcrIF12	I-F	Unknown	*P. aeruginosa* mobile element	24
AcrIF13	I-F	Unknown	*Moraxella catarrhalis* prophage	24
AcrIF14	I-F	Unknown	*Moraxella phage* Mcat5	24
AcrIIA1	II-A	Unknown	Listeria monocytogenes prophage J0161a	19
AcrIIA2	II-A	DNAbinding	*L. monocytogenes prophage* J0161a	19
AcrIIA3	II-A	Unknown	*L. monocytogenes* prophage SLCC2482	19
AcrIIA4	II-A	DNAbinding	*L. monocytogenes* prophage J0161b	19
AcrIIA5	II-A	Unknown	Streptococcus thermophilus phage D4276	20
AcrIIA6	II-A	DNAbinding	S. thermophilus phage D1811	21
AcrIIA7	II-A	Unknown	Metagenomic libraries from human gut	27
AcrIIA8	II-A	Unknown	Metagenomic libraries from human gut	27
AcrIIA9	II-A	Unknown	Metagenomic libraries from human gut	27
AcrIIA10	II-A	Unknown	Metagenomic libraries from human gut	27
AcrIIA11	II-A	DNA cleavage	*Clostridium* sp. from human gut metagenome	28
AcrIIA12	II-A	DNAbinding	*Listeria monocytogenes* prophage	29
AcrIIA13	II-A	DNA cleavage	*Staphylococcus schleiferi* prophage	30
AcrIIA14	II-A	DNA cleavage	*Staphylococcus simulans* prophage	30
AcrIIA15	II-A	DNA cleavage	*Staphylococcus delphini* prophage	30
AcrIIA16	II-A	DNA cleavage	*Listeria monocytogenes* Plasmid	31
AcrIIA17	II-A	DNA cleavage	*Enterococcus faecalis* Plasmid	31
AcrIIA18	II-A	DNA cleavage	*Streptococcus macedonicus* prophage	31
AcrIIA19	II-A	DNA cleavage	*Staphylococcus simulans* Plasmid	31
AcrIIA20	II-A	Unknown	*Streptococcus iniae* Prophage	61
AcrIIA21	II-A	Unknown	*Streptococcus agalactiae* prophage	61

AcrIIC1	II-C	DNA cleavage	*Brackiella oedipodis* ICE	16
AcrIIC2	II-C	Guide loading	*Neisseria meningitidis* prophage	16
AcrIIC3	II-C	DNAbinding	*Neisseria meningitidis* prophage	16
AcrIIC4	II-C	DNAbinding	*Haemophilus parainfluenzae* prophage	17
AcrIIC5	II-C	DNAbinding	*Simonsiella muelleri* prophage	17
AcrIII-1		Degradation of cA4	*Sulfolobus islandicus* and others with type III sys.	33
AcrIIIB1	III-B	Csx1 RNase interference	*Sulfolobus islandicus* rudivirus 2	32
AcrVA1	V-A	DNAbinding	*Moraxella bovoculi* prophage	24,25
AcrVA2	V-A	Unknown	*M. bovoculi* prophage	24
AcrVA3	V-A	Unknown	*M. bovoculi* prophage	24
AcrVA4	V-A	DNAbinding	*M. bovoculi* mobile element	25
AcrVA5	V-A	DNAbinding	*M. bovoculi* mobile element	25
Csx27	I-B	Repress Cas13b	*Bergeyella zoohelcum*	34
AcrVIA1	VI-A	Unknown	*Leptotrichia wadei* F0279 prophage	62
AcrVIA2	VI-A	Unknown	*Leptotrichia wadei* F0279 prophage	62
AcrVIA3	VI-A	Unknown	*Leptotrichia wadei* F0279 prophage	62
AcrVIA4	VI-A	Unknown	*Leptotrichia wadei* F0279 prophage	62
AcrVIA5	VI-A	Unknown	*Leptotrichia wadei* F0279 prophage	62
AcrVIA6	VI-A	Unknown	*Rhodobacter capsulat* R121 prophage	62
AcrVIA7	VI-A	Unknown	*Leptotrichia buccalis* DSM 1135 prophage	62

Conclusion

Since their discovery CRISPR-Cas systems have emerged as potential players in many areas like gene editing, screening of libraries, epigenetic editing, diagnostics, therapeutics and many more. With their growing popularity the need for tools which control and modulate CRISPR-Cas has also greatly increased. The research in Anti-CRISPR discovery and its applications has accelerated in the recent past which shows the increasing interest in this area. With the advancements in bioinformatics tools for the discovery of new Acr protein families, it is anticipated that many new Acr families will be discovered in the near future owing to their vast distribution in prokaryotic systems. In addition to this, the exact mechanisms of many Anti-CRISPR proteins are still to be elucidated. Though the research in the field of Anti-CRISPRs has become prominent initially due to the popularity of CRISPR-Cas technologies, Acrs since then emerged as an important tool for many biotechnological applications. All that has been understood in this area is just a beginning and we expect many more exciting discoveries are yet to come in future.

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Compliance with ethical standards

Declaration of Interest: All authors declare that there exist no commercial or financial relationships that could, in any way, lead to a potential conflict of interest.

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