

ANTI CRISPR PROTEINS AS A NEW TOOL FOR SYNTHETIC BIOLOGY

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Introduction

Bacteria develop by random genetic drift (RGD) and horizontal gene transfer (HGT). HGT is a technique of lateral gene transfer that enables bacteria to receive and incorporate exogenous DNA into their genome (Brito, 2021; Mejdani, 2019; Soucy et al., 2015). Phages (bacteria-specific viruses) are a key contribution to HGT since they can effectively attach bacterial cells and deliver DNA into the cytoplasm. This vector-based technique of HGT comes with the substantial caveat of possible destruction and mortality for the bacterial host (Mejdani, 2019). That guard against phage invasion and other lateral gene transfer components microorganisms have evolved methods to destroy invading DNA sequences. One of these ways is CRISPR-Cas-related genes. However, there is a range of different CRISPR technologies they all involve an RNA-directed protein complex that may anneal to a foreign DNA and/or RNA sequence and via nuclease, capability to induce destruction (Tang et al., 2019). As hypothesized, by this method, CRISPR-Cas presents a considerable danger to phage infection (Mejdani, 2019).

To evade CRISPR-Cas systems bacteriophages code for CRISPR-Cas inhibitors termed Acr (Acrs). These Acrs are tiny phage-encoded proteins that may interact and suppress CRISPR-Cas nuclease activity, hence facilitating lateral gene transfer (Davidson et al., 2020). Whereas CRISPR-Cas mechanisms are a reflection of the co-evolution between bacteria and phages, CRISPR-Cas technologies have also been exploited for biotechnological reasons (Hampton et al., 2020). Through the RNA-directed nuclease functionality of CRISPR-Cas systems, bacterial DNA patterns may be changed, leading to novel bacterial strains that can be exploited for study.

The detection of invading nucleic acids is one of the most prevalent cellular immunity techniques in all living species. The existence of DNA in the cytoplasm can stimulate the cGAS–STING signaling mechanism in eukaryotes, resulting in innate immunity induction via the generation of type I interferons¹ (Jia & Patel, 2021). Pathogens have adapted a variety of strategies to resolve such defenses, which is unsurprising (Cheng et al., 2020; Eaglesham & Kranzusch, 2020). To circumvent antiviral mechanisms, phages have developed a variety of counter techniques including alteration of limitation areas or destruction of restriction-modification cofactors (Hampton et al., 2020). The choice for point mutations in the CRISPR–Cas aimed sequence (Deveau et al., 2008) or the establishment of nucleus-like structures (Malone et al., 2020; Mendoza et al., 2020) that protect target identification or the production of Acr proteins that instantly neutralize the CRISPR–Cas effector complex (Bondy-Denomy et al., 2013; Samson et al., 2013). CRISPR–Cas processes are found in around 85 % of archaeal and almost 40 % of bacterial genomes that have been entirely sequenced (Makarova et al., 2020). Prominently, CRISPR–Cas technologies are varied, and they are nowadays divided into two broad categories, each of which contains six different kinds based on phylogenetic analysis, cas gene structure, and method of action (Makarova et al., 2015; Makarova et al., 2020). Multisubunit effector complexes make up class 1 systems (that comprise types I, III, and IV), while single-subunit effector

proteins make up class 2 methods (that contain types II, V, and VI). Despite these variances, all mechanisms follow the same fundamental action principle, which is separated into three phases: adaptation, expression, and interference (Marraffini, 2015; Van Der Oost et al., 2014). Small DNA segments originating from the attacker, termed as spacers, are incorporated into the host CRISPR locus to immunize the host cell during the initial, adaption stage; this inclusion of spacers produces a genetic record of the disease. The CRISPR loci are translated into precursor CRISPR-derived RNAs (crRNAs) and then converted into short mature crRNAs in the second stage of protection. Individual crRNAs join with Cas proteins to create crRNA–effector complexes during the destination interference phase, that are accountable for sequence-particular identification and destruction of invading nucleic acids. By experiencing rapid mutation, phages can avoid CRISPR–Cas immunity by requiring complementarity between crRNAs and phage nucleic acid goals (Andersson & Banfield, 2008; Deveau et al., 2008). Phages have developed protein blockers of CRISPR–Cas networks, termed as Acr proteins, to resist the adaptive nature of CRISPR–Cas monitoring complexes, that were initially found in 2013 (Bondy-Denomy et al., 2013). Acr have been identified for five CRISPR–Cas subtypes, comprising class 2 types II, V, and VI, as well as class 1 types I and III, with none found for type IV yet. Different techniques have been used to uncover distinct Acr that have no sequence characteristics in common. Biochemical and structural analyses have discovered particular pathways of activity for 29 Acr, revealing four main techniques for combating CRISPR–Cas processes: inhibiting CRISPR–Cas complicated assembly, attempting to block target interaction, preventing target breakage, and degrading cyclic oligonucleotide sensing molecules. Cas suppression via dimer synthesis and post-translational alteration of Cas effectors are two further developing Acr methods (Davidson et al., 2020; Hwang & Maxwell, 2019; Marino et al., 2020). CRISPR–Cas technologies have been reinvented as strong genetic modification techniques for gene and genome editing (Cong et al., 2013; Jinek et al., 2012; Mali et al., 2013), gene regulation (Qi et al., 2013), imaging, and molecular diagnosis (Adli, 2018; Hsu et al., 2014), due to their RNA-guided, sequence-specific recognition capabilities. Gene manipulation has evolved from a basic scientific technique to a technology employed in a variety of medicinal applications due to its ease and resilience.

Discovery of CRISPR–Cas inhibitors

The 1st lively CRISPR–Cas inhibitor was found in an almost similar class to the *Pseudomonas* species phage. Despite possessing promoter region regions that really should have been recognized by an updated type I F CRISPR–Cas system (Pawluk et al., 2017), those phages were capable of infecting and replicating with in *Pseudomonas* strains with just as explored I F CRISPR–Cas system⁸ (Meaden et al., 2021). Sequence analysis identified the chromosomal area responsible for the 'anti-CRISPR' trait along with the particular gene implicated (Pawluk et al., 2018; Touchon & Rocha, 2010). Five different proteins (AcrF1, AcrF2, AcrF3, AcrF4, and AcrF5) have been demonstrated to inhibit the kind I F CRISPR–Cas system (Bondy-Denomy et al., 2015). Due to the particular reason none of these proteins interfered with cas gene expression or crRNA molecule maturation, this was assumed that they prevented CRISPR–Cas interference (Marino et al., 2020). In a further investigation, four other different families of tiny proteins were displayed to suppress *P. aeruginosa* type I E CRISPR–Cas system¹⁰ (Borges et al., 2017)

Those proteins were coded by genes found among the same phage family as the class I F Acr gene (Stanley et al., 2019). Unlike the class, I F Acr molecules, not analogs of both class I E Acr amino acids have been recognized in MGEs from various genera of bacterial species (Liu et al., 2020; Peng et al., 2020). The nine previously discovered Acr protein domains did not share any system is defined features, which might lead to the recognition of supplementary Acr proteins and also peptides among other taxa or active over different CRISPR–Cas types (e Pacheco, 2019). The proteomic background of the gene producing those proteins, on the other hand, was highly similar (Derrien et al., 2012). Acr phages also produced a suspected transcription regulator known as Acr Associated (Aca1; produced by *aca1*), which featured a helix–turn–helix pattern directly ahead of something like the Acr genes (Shehreen, 2021), and an Aca regulating proteins appears to check the activity in both Acr and the *aca* alleles at optimum action even through the phage infection series (Stanley et al., 2019).

A 'guilt-by-association' bioinformatic technique was utilized in subsequent investigations to find the possible Acr gene in MGEs depending on its genomic position downstream of the probable regulator *aca1* (Yin et al., 2019). Analogous putative Acr gene was discovered upstream of genes producing a helical structure protein of different sequences from a family than Aca1 (Pawluk et al., 2018; Stanley et al., 2019). As a result of this novel Aca protein family (Aca2), additional putative Acr genes have been identified (Walker, 2017). This study discovered and functionally verified five novel groups of class I F Acr protein found in MGEs throughout the Proteobacterial phylum (Pawluk et al., 2018; Pawluk, 2016). Following that, the very first inhibitor of a types II CRISPR–Cas systems are found utilizing this guilt-by-association strategy (Davidson et al., 2020). Three small protein families (AcrIIC1, AcrIIC2, and AcrIIC3) have been demonstrated to hamper *Neisseria meningitidis* type II CRISPR–Cas9 activity (Zhu et al., 2019), both in its natural bacterial environment and when utilized like a genome-editing technology in cultured human cells (Porto et al., 2020). To find antagonists of class II A CRISPR–Cas9 systems, researchers utilized a different bioinformatic technique (Bondy-Denomy, 2018). The researchers discovered bacterial strains that had crRNAs (complementary ribosomal RNA) that matched protospacers with their own genome and generate type II-A CRISPR–Cas9 systems (Yaung et al., 2014). Due to self-targeting by both the CRISPR–Cas pathway could be assumed that it results in induction of apoptosis (Reinshagen et al., 2018), that is hypothesised that every single genome containing self-targeting would also have Acr genes in an MGEs present inside the same cell (Pinilla-Redondo et al., 2020; Yin et al., 2019).

The development of such an indigenous Acr protein of the MGEs would render the CRISPR system inactive and the cell is allowed to survive self-targeting (Hille & Charpentier, 2016). Using these criteria, 4 novel families of Acr proteins that originate to block the type II-A CRISPR–Cas pathway of *Listeria monocytogenes* 12 (AcrIIA1, AcrIIA2, AcrIIA3 also AcrIIA4) (Osuna et al., 2020). Both of such Acr proteins, AcrIIA2 and AcrIIA4, have been found to remain efficacious in contrast to the *Streptococcus pyogenes* type II-A CRISPR–Cas9 protein (Zhang & Marchisio, 2021). Acr protein is coded with genes originating in siphophages, myophages, and presumptive recombinant essentials besides pathogenicity landmasses (Leon, 2021). Except for the periodic existence of the *aca* genes just down-streams of that Acr gene, there are no shared genomic traits (Pawluk et al., 2018). Acr gene is discovered in phages at the head and the tail morphogenetic gene, as well as at the very end of their genome (Stanley et al., 2019). Many MGEs contain 2 or 3 Acr genes in a single operon,

also the significant variety in Acr gene arrangements and pairings implies that they may have been traded across MGEs (Birkholz et al., 2019; Yi et al., 2020). Acr protein is categorized established on that CRISPR–Cas system it suppresses (Marino et al., 2020). The accepted naming standard comprises the kind of system that is suppressed, a number value relating to the proteins family, also the origin of a specific Acr protein (Forsberg et al., 2021). AcrIIC1Nme, for instance, is functional at odds with the type C CRISPR–Cas systems (Thavalingam, 2019); that is the first Acr reported for that scheme, and that is expressed as an *N. meningitidis* genome as an integrated MGE (Pawluk, Amrani, et al., 2016).

Why inhibit CRISPR-Cas?

Among the eagerness and successes in Crs investigation and medical advancement, it might not be instantly understandable why is it successful to suppress the Cas proteins. Suppression, on the opposing hand, is essential for CRISPR to work properly. CRISPR is already having an impact on several seemingly unrelated and perhaps surprising features of our daily life, such as our environments (Webber et al., 2015), our foods (Scheben et al., 2017), and our health (Babačić et al., 2019; C.-H. Huang et al., 2018; Karimian et al., 2019; Lino et al., 2018). As a result, kill switch blockers that completely stop CRISPR-Cas systems may be required in a variety of conditions. The development of a conveniently given inhibitor to stop activity could be crucial for therapeutic research and, eventually, FDA agreement of some CRISPR-based drugs. Many approved drugs, such as vitamins K and prothrombin complicated concentrated for anticoagulants, for example, anticoagulant (Yasaka et al., 2002) & heparin protamine sulphate (CR, 1948), include an antiserum that can be donated to the incident of unintended abuse or to reduce the reaction. Importantly, these medications have just a short-term impact on the body, but CRISPR has a long-term effect, forming the accessibility of an emergency- stop further critical. The creation of gene drives to propagate a characteristic across a population's inhabitants or cause wild populations of species to collided totally (Hammond et al., 2016) is one of the suggested uses of CRISPR. The discovery of universally applied CRISPR inhibitors to fight unintended or intentional gene drive misuse, as well as the deploy of Crs contrary to human populations, could become a major worldwide security concern. Off-target effects (Barboni et al., 2017; Naeem et al., 2020; F. Zhang et al., 2019) are another practical reason for suppressing CRISPR. In recent work using CRISPR in the human fetus, researchers showed that disrepair division by-products may survive beyond cellular division, evolving in particular gene loss of whole chromosomes (Zuccaro et al., 2020). Inhibitors may work in two ways to reduce off-target effects. The first is timed inhibition, which prevents considerable off-target cleavage. This technique is based on the idea that on-target cleavage happens quickly because of complete guide-target complementarity, but off-target activity takes longer and folds predominantly after the on-target focus has been cut and altered. This approach was advanced by including an L7Ae: K-tum suppression mechanism to reduce Cas9 transcription and translation at the same time (Shen et al., 2019). Similar results were obtained with a timed distribution of the Acr protein AcrIIA4 (Shin et al., 2017). Sufficient enzyme inhibition is the second route through which inhibitors may reduce off-target editing. Although this is comparable to timed suppression, it usually includes the effector and inhibitor being delivered at the same time. "Off-tissue" editing is a similar notion. It is an on-the-move location: when editing is not desired in a tissue or organ, rather than an inaccurate genetic locus or target, that is altered in this situation. Unrestricted CRISPR-mediated editing exposes a variety of organs and cell types to potentially harmful off-target alterations, such as loss of large genomic

sequences or chromosomal rearrangements. that may not be illness relevance (Kosicki et al., 2018). If at everything is possible, off-tissue modification should be prohibited.

Acr proteins

Acr proteins are expressed by phages to aid phages to escape bacteria and archaea CRISPR-Cas systems. Acrs were first discovered in *P. aeruginosa* phages as a collection of five genes (157), and they are also expressed by genetic elements in a few bacteria (Pawluk et al., 2014). They were able to infect *P. aeruginosa* cultures by inhibiting the bacterium's type I-F CRISPR-Cas defensive mechanism. Experiments using translationally inept copies of the genes demonstrated that suppression was translation-based, suggesting a protein-based mechanism. Soon later, the same group discovered that few of these phages included blockers of *P. aeruginosa* type I-E mechanisms (Pawluk et al., 2014). Overall of the Anti CRISPR proteins known till 2017 targeted class I systems. Cas9 and Cas12 enzymes, on the other hand, are developed from class 2 systems and have been widely employed in biotechnology. 3 Acrs that suppress the type 2-C systems of *Neisseria meningitidis* and *Brackiella oedipodis* were the first described instances of a class 2 Acr (Pawluk, Amrani, et al., 2016). *Listeria monocytogenes* phages have Acr-encoding genes that block type II-A system is a system, such as the well-known SpCas9. Despite the fact that LmoCas9 and SpCas9 have only 53% sequence similarity, AcrIIA2 and AcrIIA4 generated proteins that could block target binding in SpCas9, proving that AcrIIA2 and AcrIIA4 are broad-spectrum inhibitors of type II-A Cas9 orthologs (Rauch et al., 2017). AcrIIA2 and AcrIIA4 are shown to inhibit SpCas9 in human cells in the same investigation. A fifth type II-A inhibitor was found. this time from *Streptococcus thermophilus* phages, using a strategy that focused on naturally existing Acr activity in phage strains to find possible Acrs. Later, AcrIIA5 was discovered to block all types II Cas9s, including all Cas9 homologs utilized in genome engineering. While no inhibition of type 2-B *Francisella novicida* (Fn)Cas9 was seen in plaque experiments (Garcia et al., 2019). AcrIIA5-dependent suppression of FnCas9 was reported in vitro (Song et al., 2019; Watters et al., 2020). Using a guilt-by-association technique, three novel proteins. AcrIIA13-15, was recently discovered as exclusively inhibiting *S. aureus* (Sa) Cas9 but not SpCas9 in living cells (Watters et al., 2018; Watters et al., 2020). SaCas9 inhibitors are a useful addition to the CRISPR-Cas9 toolkit because of SaCas9's modest size and relative simplicity of administration. Another extensively utilized Cas enzyme, type V Cas 12a, has also been shown to contain Acr inhibitors, Acrs, also known as AcrVALI-5, was discovered to impede self-targeting by Cas12a systems in two separate investigations (Watters et al., 2018).

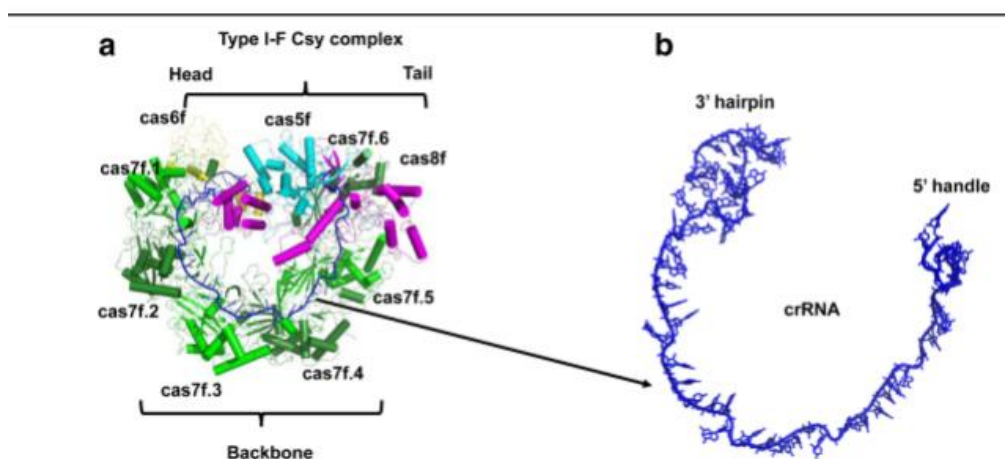
Structure of Acr proteins

Framework biologist's research on Acr proteins has solved the geometries of Acr proteins or different fields of activity such as interaction with target CRISPR-Cas effectors. The structures of 7 Acr proteins have been revealed: AcrF1, 2, 3, 10, AcrIIA1,4, and AcrIIC1. Though, there is still a need for a systematic and comprehensive theoretical design of antiCRISPR amino acids.

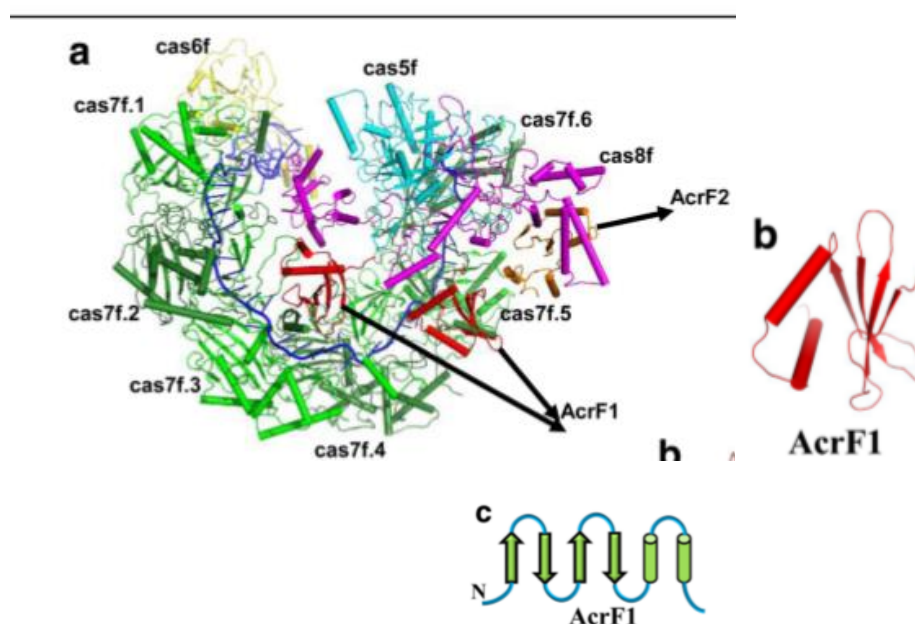
Structure of AcrF1

Choudhury and colleagues first discovered that the electron microscopy structure of a subtype I-F Csy molecule related to two unique Acr proteins, AcrF1 or AcrF2, with just an average resolving of 3.4 Å was linked to two distinct Acr proteins, AcrF1 or

AcrF2 (Chowdhury et al., 2017). The Cas6f head, Cas7f backbone, and Cas8f-Cas5f tail make up the overall structure of the type I-F Csy complex, which has a nearly closed ring topology. The 60-nucleotide crRNA, which resembles a string and tethers the complex's protein subunits together, plays an important structural role in compound formation. Gene 35 of the *Pseudomonas aeruginosa* phage JBD30 encodes the 78-amino-acid amino acid AcrF1. The general structure of AcrF1 is fairly simple, divided into 4 anti-parallel strands and two anti-helices ($\beta 1 \uparrow$ - $\beta 2 \downarrow$ - $\beta 3 \uparrow$ - $\beta 4 \downarrow$ - $\alpha 1$ - $\alpha 2$). These 4 anti-parallel -strands create a -sheet that binds to the 2 anti -helices at the C-terminus to produce a hydrophilic core (Maxwell et al., 2016).



Structural view of Type I-F Csy complex (a)Complex of type IF Csy structure The type IF Csy complex's cassf, cassif, cast, and cassf subunits are colored cyan, yellow, green, and magenta, respectively. The crRNA is blue in colour. (b)An expanded image of the crRNA structure, which looks like a string.



Structural view of type I-F Csy complex bound to AcrF1/2 .(a)The type I-F structure Two Acr proteins, AcrF1 and Acr F2, were bound by the Csy complex. The type I-F Csy complex's cas5f, cas6f cas7f, and cas8f subunits are coloured as shown in Fig.

1a. The structures AcrF1 and AcrF2 are coloured red and orange, respectively. (b) Also, topological perspective. (c) 3D structure of AcrF1

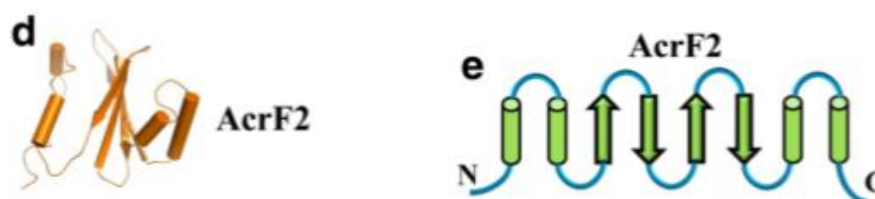
Table 1. Anti –CRISPR protein and their mechanism of action

Anti CRISPR (sources)	Size (amino acids)	CRISPR inhibited	Inhibition mechanism	Structure (PDB code)	References
AcrE1 (<i>P.aeruginosa</i>)	100	Type I-E	—	—	(Pawluk et al., 2014)
AcrE2 (<i>P.aeruginosa</i>)	84	Type I-E	—	—	(Pawluk et al., 2014)
AcrE3 (<i>P.aeruginosa</i>)	68	Type I-E	—	—	(Pawluk et al., 2014)
AcrE4 (<i>P.aeruginosa</i>)	52	Type I-E	—	—	(Pawluk et al., 2014)
AcrF1 (<i>P.aeruginosa</i>)	78	Type I-F	Inhibits DNA binding	2LW5,5UZ9, 6ANV,6B46, 5UZ9,6B47	(Bondy-Denomy et al., 2013; Chowdhury et al., 2017; Guo et al., 2017)
AcrF2 (<i>P.aeruginosa</i>)	90	Type I-F	The binding site of dsDNA is partially overlapped.	5GNF,5GQH, 5B71	(Bondy-Denomy et al., 2013; Chowdhury et al., 2017; Guo et al., 2017)
AcrF3 (<i>P.aeruginosa</i>)	139	Type I-F	The entrance to the DNA binding tunnel is blocked, preventing fresh sequence acquisition.	—	(Bondy-Denomy et al., 2013; X. Wang et al., 2016)
AcrF4 (<i>P.aeruginosa</i>)	100	Type I-F	—	—	(Bondy-Denomy et al., 2013)
AcrF5 (<i>P.aeruginosa</i>)	79	Type I-E/F	—	—	(Bondy-Denomy et al., 2013)
AcrF6 (<i>P.aeruginosa</i>)	100	Type I-F	—	—	(Pawluk, Amrani, et al., 2016)
AcrF7 (<i>P.aeruginosa</i>)	67	Type I-F	—	—	(Pawluk, Amrani, et al., 2016)
AcrF8 (<i>P.atrosepticum</i>)	92	Type I-F	—	—	(Pawluk, Amrani, et al., 2016)

AcrF9 (<i>V.parahaemolyticus</i>)	68	Type I-F	—		(Pawluk, Amrani, et al., 2016)
AcrF10 (<i>S.xiamenensis</i>)	97	Type I-F	DNA mimic blocks DNA binding	6ANW,6B48	(Guo et al., 2017; Pawluk, Amrani, et al., 2016)
AcrIiA1 (<i>L.monocytogenes</i>)	149	Type II-A	—	5Y6A	(Ka et al., 2018; Rauch et al., 2017)
AcrIiA2 (<i>L.monocytogenes</i>)	123	Type II-A	Inhibits DNA binding	—	(Rauch et al., 2017)
AcrIiA3 (<i>L.monocytogenes</i>)	125	Type II-A	—	—	(Rauch et al., 2017)
AcrIiA4 (<i>L.monocytogenes</i>)	87	Type II-A	PAM mimic inhibits DNA binding by interacting with the active site inside the RuvC domain and preventing the HNH domain from changing conformation.	5XBL, SVW1,5VZL	(Dong et al., 2017; Rauch et al., 2017; Shin et al., 2017)
AcrIiA5 (<i>S.thermophilus</i>)	140	Type II-A	—	—	(Hynes et al., 2017)
AcrIiC1 (<i>N.meningitidis</i>)	85	Type II-C	Protects the catalytic core by binding the HNH domain.	5VGB	(Harrington et al., 2017; Pawluk, Amrani, et al., 2016)
AcrIiC2 (<i>N.meningitidis</i>)	123	Type II-C	—	—	(Pawluk, Amrani, et al., 2016)
AcrIiC3 (<i>N.meningitidis</i>)	116	Type II-C	Cas9 dimerization is induced, which hinders DNA binding.	—	(Harrington et al., 2017; Pawluk, Amrani, et al., 2016)

Structure of AcrF2

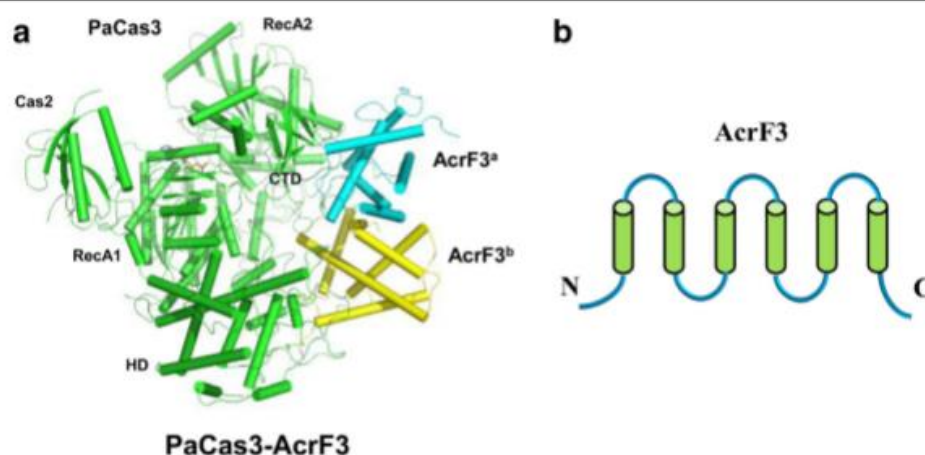
AcrF2, a small alkaline protein, is a 90-amino acid protein produced by *P. aeruginosa* phage D3112 via nucleotide Thirty. The geometry of AcrF2 coupled to the kind I-F Csy molecule was identified by using a single-particle cryo-electron microscope. AcrF2's logical narrative is sandwiched, with 4 antiparallel α -strands flanked on each side by two β -helices ($\alpha 1,2$ - $\beta 1,4$ $\uparrow$ - $\beta 2,3$ $\downarrow$ - $\alpha 3,4$) except for 2-helices at the N-terminus, AcrF2 has a topological structure that is quite similar to AcrF1 (Chowdhury et al., 2017; Guo et al., 2017).



(d) AcrF2 topological view (e) AcrF2 helices and strands are shown by green cylinders and arrows, respectively.

Structure of AcrF3

AcrF3 is a bigger protein than AcrF1 or AcrF2 and is coded by gene -35 from the *P. aeruginosa* phage JBD. It is greater than AcrF1,2 and has 139 proteins (Table 1). Zhu's group discovered the crystalline structure of AcrF3 in combination with PaCas3 in 2016, with clarity of 2.6 Å (Mallon & Bailey, 2016; X. Wang et al., 2016). Wang and colleagues then utilized a cryo-EM single-molecule technique to solve a 4.2-residue PaCas3 (residue 106–1076)–AcrF3 complex using the molecular structure of AcrF3 with a resolution of 1.5Å. The all framework of AcrF3 consists of 6 -helices. The gel filtration chromatographic findings indicate that AcrF3 has a dimeric structure. The HD domain range is RecA1, RecA2, and PaCas3's CTD form a groove around the AcrF3 dimer. PaCas3 and AcrF3 form a dense complex with many H-bonds and a significant hydrophobic relationship (J. Wang et al., 2016).



Structure of PaCas3 in complex with AcrF3 (a)PaCas3 is coloured green in this structure of the PaCas3-AcrF3 complex. Respectively, the AcrF3 dimer is coloured cyan and yellow. Cas2, RecA1/2, HD, and CTD domains of PaCas3 have been tagged. (b)AcrF3 topology.

Structure of AcrIIA4

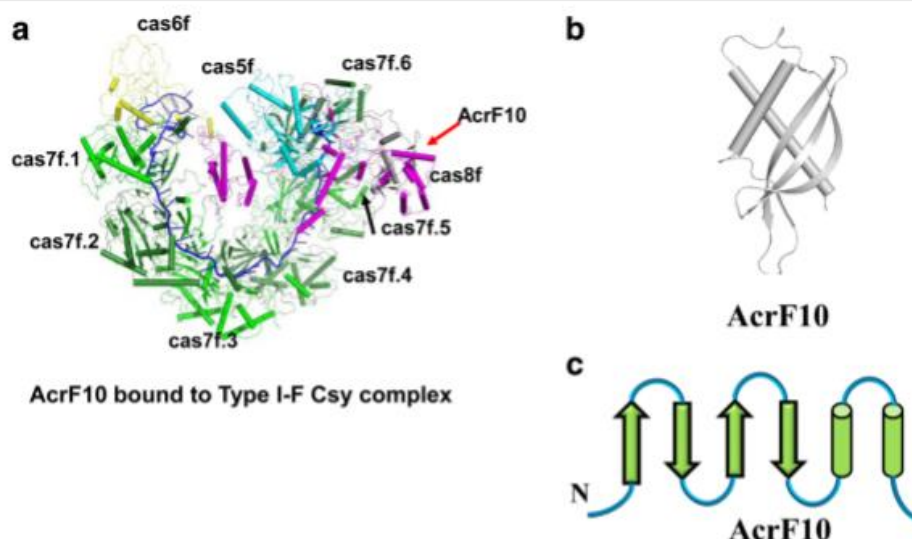
AcrIIA4 does have a "triangle" bind that consists of 3 anti-parallel strands and 3 helices ($\alpha 1\text{-}\beta 1\uparrow\text{-}\beta 2\downarrow\text{-}\beta 3\uparrow\text{-}\alpha 2\text{-}\alpha 3$). These 3 anti-parallel strands create a β -sheet containing three α -helices over one end. AcrIIA4 has the same topological structure as AcrF1, but the B-strand has replaced the Alpha-helix Stand. The PAMinteracting region in the AcrIIA4–SpyCas9–sgRNA structures are held by AcrIIA4, which binds to the spyCas9 TOPO, CTD, and RuvC domains (Dong et al., 2017).

Structure of AcrIIA1

A common type II-A Acr is AcrIIA1, a 149-amino-acid Acr derived from an *L. monocytogenes* prophage. AcrIIA1's geometry was recently established using X-ray diffraction with a magnification of 2.0Å. Two AcrIIA1 compounds combine to produce a homodimer, each with an all-helical 2-domain architecture (Ka et al., 2018).

Structure of AcrF10

X-ray diffraction was used to determine the structure of AcrF10 (Guo et al., 2017).



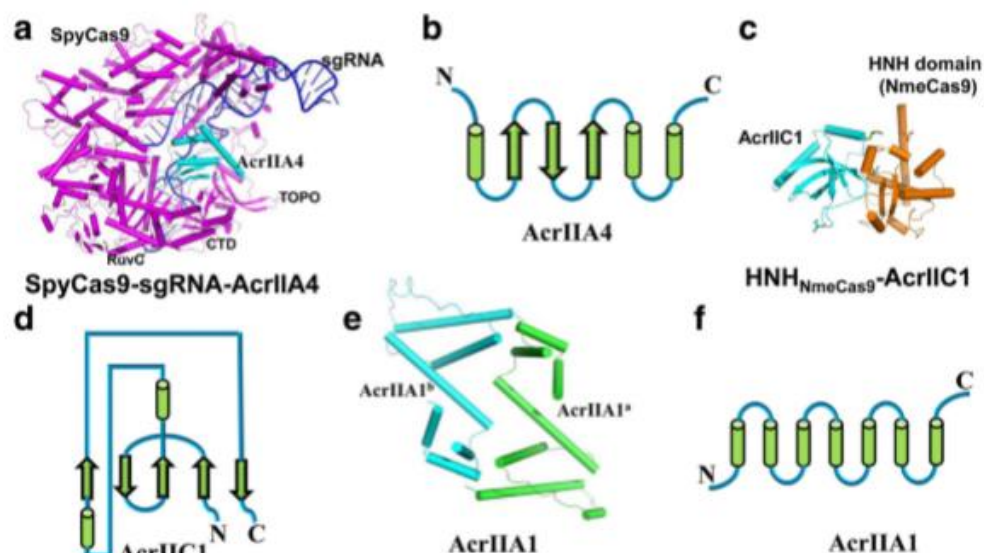
View of the I-F Csy complex linked to AcrF10 from a structural standpoint (a) Csy complex of type I-F connected to AcrF10 structure. The type I-F Csy complex's cas5f, cas6f, cas7f, and cas8f subunits are colored as shown in the 3D structure of AcrF10. AcrF10 (b) and topological view (c).

Structure of CrIIA4

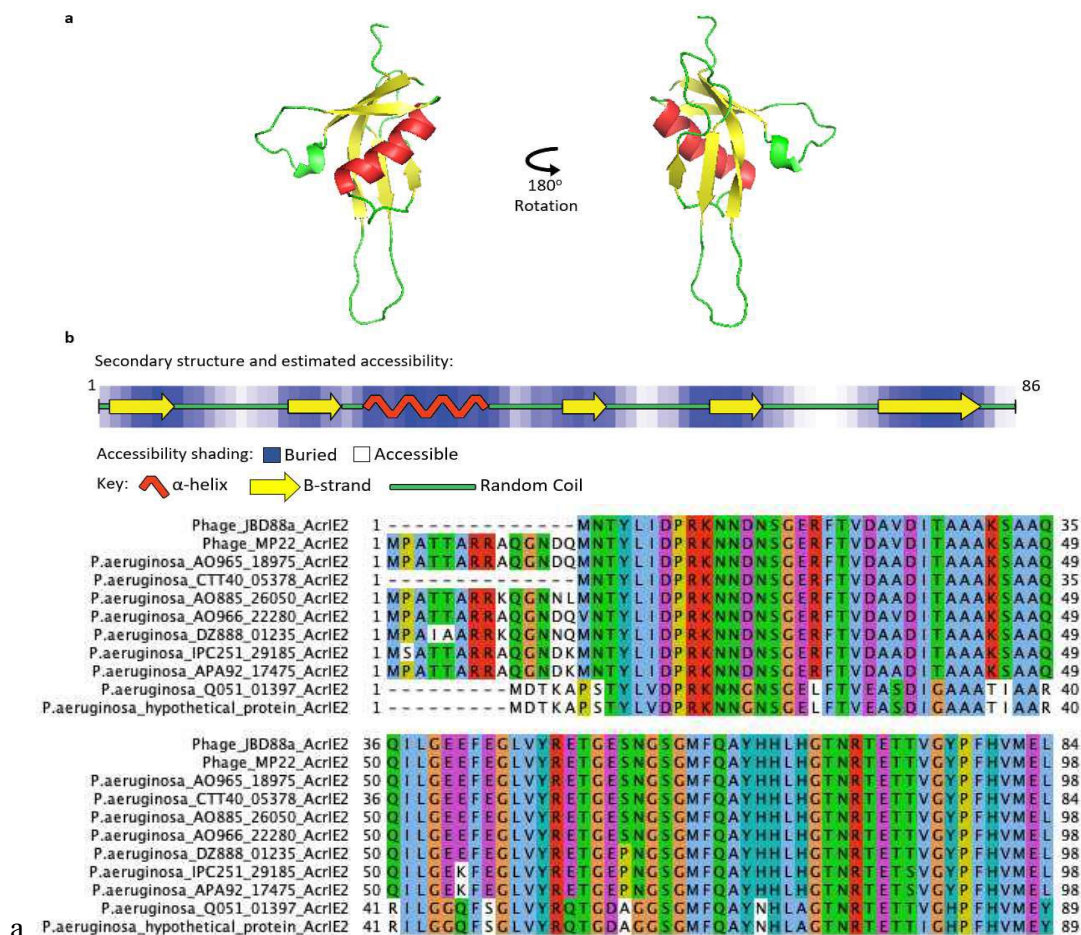
CrIIA4 is a bacteriophage from *L. monocytogenes* that contains 87 amino acids.

Structure of AcrIE2

The structure identified for AcrIE2 is depicted in Figure 9a. The structure of AcrIE2 is an 84-residue amide with a core alpha-helix and a five-strand antiparallel beta-sheet (Figure 9a, b). The α -helix packs against one side of the β -sheet, generating a compact hydrophobic core with 10 residues >90 percent buried. The geometry of AcrIE2 is not related to any previously known Acr protein, and research utilizing the Dali server did not indicate structural similarities to another protein (Holm & Rosenström, 2010).



Type II Acr proteins seen structurally. In the AcrIIA4-SpyCas9-sgRNA complex, SpyCas9 and AcrIIA4 are magenta and cyan, respectively. Blue is the color of crRNA. RuvC CTD and TOPO domains of SpyCas9 have been identified. (b) AcrIIA4 from a topological standpoint. AcrIIC1 and the HNHnmeCas9 domain are colored cyan and orange, respectively, in this structure of the AcrIIC1-HNHnmeCas9 domain complex. AcrIIC1 from a topological standpoint (e) AcrIIA1 dimer structure with its cyan and green colored IDs. (f) Kf AcrIIA1 topological perspective.



AcrIE2 NMR structure (Figure 9). (a) The backbone atoms in ribbon representation (N, C, and C') in the NMR solution structure of AcrIE2. (b) AcrIE2 secondary structure with different shades of blue indicating relative residue accessibility. Blastp alignment of the JBD88a-AcrIE2 protein's amino acids.

Inhibition mechanisms of Acr proteins

AcrIIA1-A21

Listeria monocytogenes prophages encode AcrIIA1-A4, which suppresses the type II-A CRISPR-Cas system among *L. monocytogenes* strain (Rauch et al., 2017). The seeming self-targeting of CRISPR-Cas systems in *L. monocytogenes* prompted about finding of AcrIIA1-A4. this bacterial genome demonstrates the coexistence of the protospacers (derived from MGE) and excellent matching spacer, which is thought to be accountable for apoptosis when the CRISPR array is expressed. As a result, a bioinformatics technique was used to search *L. monocytogenes* DNA for a specific inhibitor — of that viral origin — which may disable this CRISPR-Cas system resulting in enabling cell viability (Rauch et al., 2017). The mostly co of *acrIIA1* with different *acrIIA* genes (for example AcrIIA2-A4 also AcrIIA12 (Osuna et al., 2020; Rauch et al., 2017) identified in *L. monocytogenes* prophage revealed how AcrIIA1 suppresses gene editing from LmoCas9, implying that AcrIIA1 needs an Acr companion for inactivation of Cas9. Cas9 disintegration is aided by AcrIIA1, whilst DNA attachment is hindered by the other Acr. As a result, the collaboration of the two Acr is required to efficiently disable Cas9 at the time of the invasion of phage (Osuna et al., 2020). AcrIIA1 has been found to build two helical-domain Dimer architectures and a motif of helix-turn-helix (HTH) at the N terminus (Ka et al., 2018). When AcrIIA1 is highly expressed in *E. coli*, it may also bind with RNAs (Ka et al., 2018). In *E. coli* and human cells, AcrIIA1 did not demonstrate any inhibition of SpyCas9-guided gene editing (Rauch et al., 2017), but it did show strong inhibitory effectiveness in *Saccharomyces cerevisiae* (Nakamura et al., 2019).

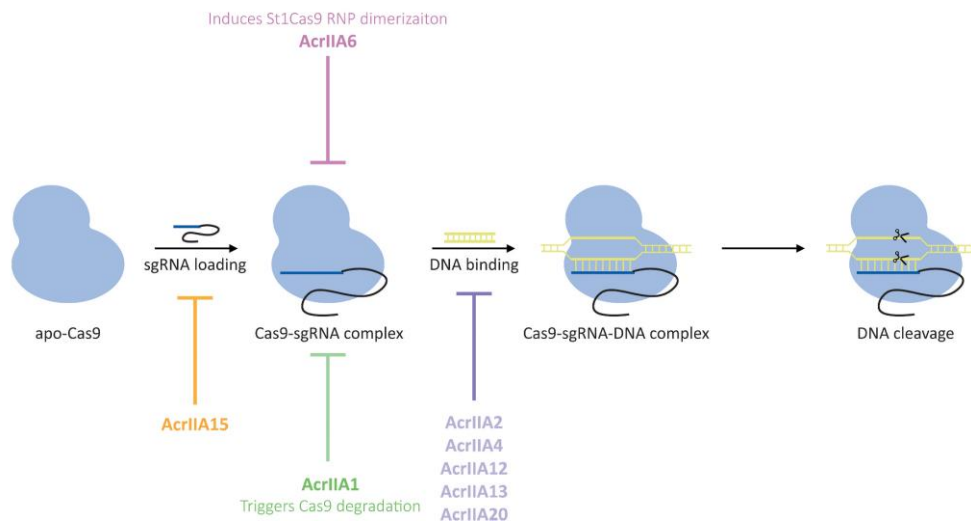
Among human cells, *E. coli*, and also *S. cerevisiae*, AcrIIA2, and AcrIIA4 act well against SpyCas9-driven DNA cleavage (Basgall et al., 2018; Nakamura et al., 2019; Rauch et al., 2017). AcrIIA4 inhibits SpyCas9 nuclease action fully (Rauch et al., 2017), but AcrIIA2 inhibits SpyCas9 nuclease function less effectively in mammalian cells than in *S. cerevisiae* (Nakamura et al., 2019). Modification to AcrIIA2, as attaching the GFP (green fluorescent protein) to the N or C terminals, seems to significantly reduce its inhibitory capacity, while AcrIIA4 appears to be more resistant (Basgall et al., 2018). in their unbound form AcrIIA2 and AcrIIA4 are monomers, unlike AcrIIA1 (Yang & Patel, 2017). AcrIIA2 and AcrIIA4 have a functional similarity in that they both use DNA mimicry to suppress the type II-A CRISPR-Cas mechanism. AcrIIA2 and AcrIIA4 have a greater affinity for the SpyCas9-sgRNA dual complex than DNA when produced in bacterial cells. They engage SpyCas9-sgRNA as a result block their site of DNA-binding (Dong et al., 2017), inhibiting the recognition of PAMs and so the development of the SpyCas9-sgRNA-DNA ternary complexes. Subsequently, a SpyCas9-sgRNA-DNA ternary complex is formed, even not AcrIIA2 not AcrIIA4 could remove the DNA of that the SpyCas9-sgRNA complexes. In the lack of sgRNA, AcrIIA2 and AcrIIA4 do not interfere with SpyCas9. SpyCas9 does undergo a conformational shift after engaging the sgRNA, which produces the attaching furrow of the DNA substrate or Acr proteins (Dong et al., 2017; Jiang et al., 2015; Jinek et al., 2014; Yang & Patel, 2017). the structural modifications generated by AcrIIA2 and AcrIIA4 are somewhat different. AcrIIA2

establishes a hydrogen bond (H) with the PI (PAM-interacting), HNH (His-Asn-His), WED (wedge-like), and REC2 (RNA be familiar with) domain of that SpyCas9 when it links SpyCas9-sgRNA (Liu et al., 2019). AcrIIA4 forms hydrogen bonds with the PI, WED, and RuvC domains rather than the HNH domain of SpyCas9 (Dong et al., 2017; Liu et al., 2019). A nuclease domain HNH and RuvC are essential for that SpyCas9-dependant RNP action. The former cleave the non-target strands of the dsDNA substrates, The former cleaves the target strand, but the latter does not (Jinek et al., 2014) That after the DNA substrate has indeed placed against SpyCas9-sgRNA besides base-pairing among DNA substrate also sgRNA has been finished, an HNH domain experiences a conformation reorganization which is hypothesized for promoting the inactivity to vigorous alignment transition (Jiang et al., 2015). The hydrogen bond(H) in between AcrIIA2 and the HNH domains of SpyCas9 prevents any endorsement change in HNH, preventing the RNP from acting as a nuclease. In comparison, AcrIIA4 interacts along with the RuvC dominion active site and prevents it. (Dong et al., 2017; Jiang et al., 2015; Shin et al., 2017; Yang & Patel, 2017), provide comprehensive research on both construction of SpyCas9-sgRNA-AcrIIA2 and also of the SpyCas9-sgRNA-AcrIIA4. The aggressive phages of *Streptococcus thermophilus* encode AcrIIA5 and AcrIIA6 (Hynes et al., 2018; Hynes et al., 2017). AcrIIA5 is anticipated to have a unique motif which is coiled-coil which is thought to operate as a binding motif of nucleic acid, but AcrIIA6 should not (Hynes et al., 2018; Hynes et al., 2017).

So far, only a few research have looked into the process of AcrIIA5 inhibition (An et al., 2020; Garcia et al., 2019; Hynes et al., 2017; Song et al., 2019). However, it's unclear whether AcrIIA5 works via cleaving sgRNA (Garcia et al., 2019) or by inhibiting RuvC domain nuclease action (Song et al., 2019). AcrIIA5 has been shown to correlate to the Cas9-sgRNA than apo-Cas9 (i.e. short of sgRNA) in a new analysis, suggesting the Acr proteins might use an initially unidentified approach to restrict a variety of Cas9 homologs (that makes usage of its N-terminal fundamentally muddled region – IDR) (An et al., 2020). AcrIIA5 overpowers genetic excision intermediated by several Cas9 homologs after type II-A (e.g., SpyCas9), type II-B (e.g., Francisella novicida Cas9 – Fn AcrIIA6 creates a dimer with two St1Cas9-binding sites that are homologous. AcrIIA6 inhibits St1Cas9 by causing St1Cas9 RNP dimer formation by intermolecular contacts (Fuchsbaauer et al., 2019). As a result, the AcrIIA6 dimer lowers the St1Cas9 RNP's interaction efficiency for the aim DNA. AcrIIA7-A10 were identified using a unique in elevation amount transmission method relying on a mock genetic track in E. coli cells, anywhere the action of fresh Acr protein is linked to antibiotic choice. Human gut metagenomes had AcrIIA7, AcrIIA8, and AcrIIA9, whereas a soil metagenomic collection contained AcrIIA10. These four Acrs' operating mechanism is currently unclear. AcrIIA7, on the other hand, is thought to work in a novel manner, disrupting the assembly of the SpyCas9-sgRNA binary complexes (Uribe et al., 2019). AcrIIA11 is discovered comparably, using protein efficient action and high-throughput metagenomic library transmission (Forsberg et al., 2019). However, the mechanism by which AcrIIA11 performs its role remains uncertain. This suggests that AcrIIA11 interacts with a SpyCas9 area which conformation does not vary whether SpyCas9 forms complexes with equally the solitary sgRNA or both the sgRNA and the target DNA. Furthermore, when AcrIIA11 binds SpyCas9, it may experience a conformational shift which made it more likely to attach dsDNA (Forsberg et al., 2019). As previously reported, AcrIIA12 was discovered in *L. monocytogenes* prophage, wherever it co-occurs with AcrIIA1. Cas9 is likely prevented from attaching to DNA by AcrIIA12. There are preserved HTH-

encoding *aca* in the proximity of *acr* genes, which is significant. This discovery led to the hypothesis that the presence of HTH might indicate the presence of *acr* genes (Bondy-Denomy et al., 2015; Pawluk, Staals, et al., 2016; Stanley et al., 2019). A bioinformatics technique known as 'guilt-by-association' has been developed based on this concept to find possible *acr* genes in a variety of bacterial species (Bondy-Denomy et al., 2013; Pawluk, Amrani, et al., 2016). The anticipated HTH motif is retained in the N-terminal region of all three Acrs. They do, however, have distinct C-terminal dominions which play a role in the Acr action that disrupts SauCas9's function. The SauCas9-sgRNA complex is prevented from attaching to the target DNA by AcrIIA13, while sgRNA loading onto SauCas9 is hampered by AcrIIA15. AcrIIA14 doesn't prevent sgRNA packing or target DNA engagement, but it does reduce SauCas9 DNA breakage (Watters et al., 2020). In human cells, AcrIIA13 and AcrIIA14 are particularly effective suppressors of SauCas9-guided gene-altering, with AcrIIA13 demonstrating the greatest efficacy (almost complete suppression). AcrIIA15's inhibitory effectiveness is substantially lower than AcrIIA13-A14's. This was prompted by the discovery that *acrIIA1*, which contains the HTH motif, was often found down-streams of *acrIIA2-A4* [38]. Although its action method has yet to be determined, AcrIIA16-A19 had the capacity to block the activity of SpyCas9 and stop DNA breakage in humanoid cell. Significantly, AcrIIA16 and AcrIIA17 inhibit type II Cas9 homologs throughout a wide range. Machine-learning methods had recently being used for the search for new Acr (Eitzinger et al., 2020; Gussow et al., 2020). AcrIIA20 is the smallest type II Acr discovered so far, with just 64 amino acids. In vitro, AcrIIA20 was shown to be a strong SinCas9 inhibitor over DNA mockery, although it was fewer efficient in contradiction of SpyCas9. AcrIIA21 was very effective against SinCas9 nuclease movement, still only moderately effective against SpyCas9 and SauCas9. Its methods of operation are yet unclear (Eitzinger et al., 2020).

Anti-CRISPR Type II-A Inhibitory Mechanisms

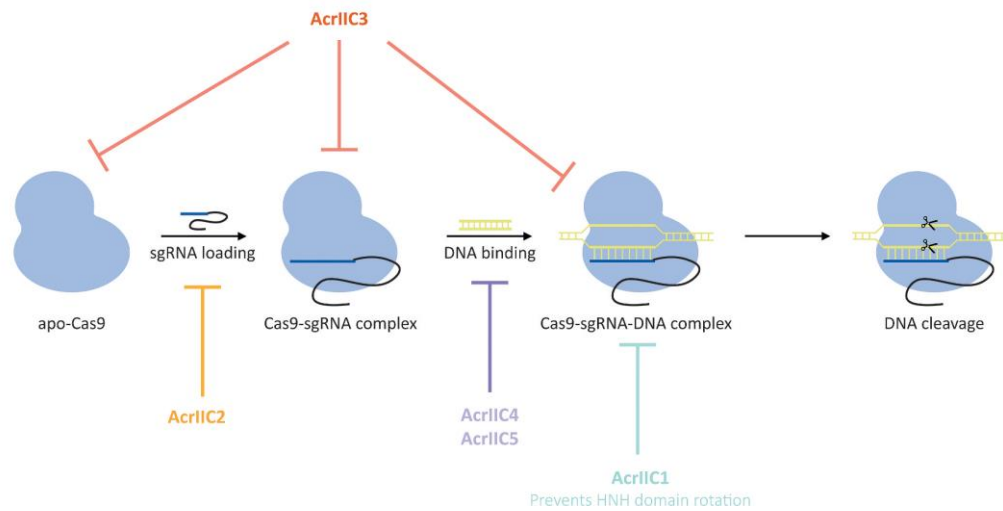


AcrIIC1-5

Using the 'guilt-by-association' bioinformatics technique, 5 types of II-C Acr protein called AcrIIC1-C5, are found then are characterized so far (Lee et al., 2018; Pawluk, Amrani, et al., 2016). AcrIIC1-C3 was detected within *N.meningitidis* and MGE, while AcrIIC4 too AcrIIC5 were detected in *Haemophilus parainfluenzae* then also in *Simonsiella muelleri* strain, respectively. Nme1Cas9 which is also present in humanoid cells (Lee et al., 2018; Pawluk, Amrani, et al., 2016), HpaCas9, also

SmuCas9 both of them are prevented from cleaving DNA by each five type II-C Acrs. AcrIIC1-C4 is efficient against Nme2Cas9, a homolog of Nme1Cas9 (Edraki et al., 2019; Lee et al., 2018; Pawluk, Amrani, et al., 2016). (two NmeCas9s differently, essential, just for the PI domains, that result in different PAM arrangement recognitions). On the other hand, AcrIIC5 performs best at Nme1Cas9 (Lee et al., 2018; Pawluk, Staals, et al., 2016). Other type II-C Cas9 homologs, like GeoCas9 and CjeCas9, are likewise inhibited by AcrIIC1. As a result, it is classified as a broad-spectrum inhibitor of type IIC (Harrington et al., 2017). It also engages Cas9 analogs by creating hydrogen bonds along with its largely preserved HNH nuclease domains. The HNH nuclease domain's conformational variations are limited by these hydrogen bonds, which reduces DNA breakage while allowing the loading of RNA and binding of DNA (Harrington et al., 2017). When the Cas9-sgRNA complex of surveillance engages the DNA substrate, the HNH nucleases domains rotate and the conformational shift to the catalytically activated state occurs. The target-strand cleavage is preconditioned by a catalytically active HNH, which leads to RuvC nuclease domain initiation. Both strands' target and non-target breakage is eventually achieved by the two catalytically active nuclease domains (Harrington et al., 2017; Jinek et al., 2014; Sternberg et al., 2015; Sun et al., 2019). The HNH nuclease domains are no longer able to spin after AcrIIC1 engagement, keeping Cas9 inactive (Harrington et al., 2017). AcrIIC3 is also engaged by the HNH nucleases domains of Nme1Cas9 as a monomer and inhibits their rotations (Sun et al., 2019). Furthermore, DNA-binding is not entirely abolished but just decreased following the synthesis of Nme1Cas9-sgRNA-AcrIIC3 (Harrington et al., 2017; Sun et al., 2019). Now Two AcrIIC3 monomer capture which causes the dimer formation of two different Nme1Cas9s in more detail. Every AcrIIC3 monomer binds to the HNH nuclease domains of a Nme1Cas9 and the REC2 domains of the second Nme1Cas9 (they are spatially opposite each other) (Kim et al., 2019; Sun et al., 2019; Zhu et al., 2019). As a component of a Nme1Cas9-sgRNA-AcrIIC3 dimer by or devoid of DNA mark, AcrIIC3 inhibits HNHs nucleases domains spinning and confines Nme1Cas9 in an inactivated state, preventing the cleavage of DNA (Sun et al., 2019). AcrIIC2 is exclusively active as a dimer and acts on Nme1Cas9 in an unusual way. The negatively charged surface of the AcrIIC2 dimer links with Nme1Cas9's positively charged arginine-rich bridging helix (BH). That hinders the loading of RNA and also the development of the Nme1Cas9-sgRNA complexes as a result. As a result, Nme1Cas9 is rendered inactive and becomes vulnerable to proteolytic enzyme or protein (Thavalingam et al., 2019; Zhu et al., 2019). AcrIIC2 has lesser reactivity for the establishing Nme1Cas9-sgRNA surveillance complex, resulting in decreased inhibition effectiveness (Thavalingam et al., 2019; Zhu et al., 2019). Surprisingly, two BH alterations (T62R and T73R) provide the same AcrIIC2-reaction site to SpyCas9 as Nme1Cas9. As a result, AcrIIC2 inhibits like SpyCas9 mutants, indicating that AcrIIC2 that interacts largely along with the BH that of Nme1Cas9 (Thavalingam et al., 2019; Zhu et al., 2019). Nonetheless, AcrIIC2 develops the stable complex (along with varying affinities) in vitro and Cas9 develop several subtypes of type II: Nme1Cas9 and CjeCas9 (II-C subtype); SauCas9 and SpyCas9 (II-A); and FnoCas9 (II-B) (II-B). In any case, only Nme1Cas9 (Zhu et al., 2019) has high inhibitory efficiency. Nme1Cas9 cannot attach to DNA when AcrIIC4 and AcrIIC5 are present (Lee et al., 2018). However, the Acr protein's structural structures are absent, and there mode of action has yet to be discovered (see Fig. 2 for an summary on Acr type II-C means of action).

Anti-CRISPR Type II-C Inhibitory Mechanisms



Acr protein metabolism

Chains of peptides were normally totally stable in solution, and nonenzymatic hydrolysis may take years (Kahne & Still, 1988). While there are several routes inside the body of a human that break down proteins, Acr protein digestion must be fairly expected if it is considered to be transcribed or translated, which is then destroyed, within cells. The autophagy-lysosomal route and the ubiquitin-proteasomal route are the two most common pathways for intracellular proteolysis. Protein is uptake into autophagic vacuole inside the cell in a nonselective manner. These entities may subsequently combine with lysosomes, which contain proteases that destroy the proteins that have been trapped (Meijer & Codogno, 2004). The ubiquitin-proteasomal mechanism, on the other hand, is more selective. In a nutshell, ubiquitin polypeptides are covalently linked to the target protein to mark it for destruction (ubiquitination). The proteasome subsequently degrades the ubiquitinated proteins. Proteolysis releases amino acids that may be utilized by biological activities, such as protein synthesis (Glickman & Ciechanover, 2002). Actually fusion related to Acrs with the cell division-controlled proteins Cdt1 was utilized to show that Cas9 inhibition through the ubiquitin-proteasomal pathway is cell cycle-dependent (Matsumoto et al., 2020). One disadvantage of using Acr protein is that the half-life of Acr protein is affected by cellular metabolic parameters for example type of cell, stress, and health of the cell.

Uses for gene-editing technologies

The usage of Acr proteins that is on leave during genetic modification is one of their most important applications. It's critical for CRISPR-Cas9 technology to have as bit off-target activity as possible, especially for therapeutic applications. Even though efforts are being made in a big way have resulted in Cas9 variations with improved specificity. extreme or severe Cas9 action may enhance the risk of off-target editing or toxicity, requiring a method to stop Cas9 action once the required outcome has been achieved. Acr proteins, when used in conjunction with modified Cas9 variations and methods to regulate Cas9 production, can provide an additional defence against Cas9's potentially harmful consequences. For example, in minimizing the interval of Cas9 function and going to take advantage of kinetics changes in Cas9 editing at on- and off-target sites (Shin et al., 2017).

The timed release of AcrIIA4 lowered the degree of off-target editing in cell inserted genes which are hereditary components that induce superMendelian heredity to spread desirable features in a population, have also been developed using CRISPR technology. The continuous advancement of female sterility-inducing gene drives in mosquitos to remove vector-borne illnesses like malaria is a good example (Nateghi Rostami, 2020). As a safety precaution, effective awareness of the distribution of a gene drive after its first discharge is a very valuable property. After the parental driving microorganisms are discharged into the essential ecosystem, Acr proteins could be used to put a stop to the proliferation of CRISPR base drive (Basgall et al., 2018).

Uses for dCas9-based applications

Various effector proteins could be connected or recruited to genomic regions of interest using nuclease-inactive dCas9. dCas9 was given recombinant proteins (FPs) as well as transcriptional stimulators and downregulation, for instance, chromosomal imaging and modulation of a specific gene can be obtained. The use of dCas9-based technologies allows not only genome manipulation but also epigenome manipulation by the fusing of DNA demethylation enzymes (e.g., TET) or histone-modifying effectors (e.g., LSD1 or p300) (Adli, 2018). Acrs which control the actions of these functional categories can likewise be utilized to inhibit DNA binding. Type II Acr proteins, for example, have been utilized to regulate chromosome labeling by dCas9-FPs and demethylation by dCas9-Tet1 fusions in created pluripotent stem cells (Basgall et al., 2018; Bubeck et al., 2018; Rauch et al., 2017). Furthermore, by modulating CRISPRi and CRISPRa, Acr proteins have permitted programmed and flexible gene regulation (Hoffmann et al., 2019; Nakamura et al., 2019).

Engineering Acr proteins:

Acr proteins can commonly survive epitope tag and FP fusion without losing their inhibitory effectiveness. Inserting an external region, A fluorescence peptide, also including mCherry, at precisely AcrIIC1 surfaces that has been chosen locations greatly increased Nme1Cas9 inhibitory (Mathony et al., 2020). This opens up the possibility of modifying Acr proteins by inserting additional domains while retaining the inhibitory action. The inducible destabilization domain, which degrades the proteins within the absence of an exterior binding referred to as Shield1, was fused to Acr proteins to achieve post-translational regulation From a synthetic biology standpoint, (Nakamura et al., 2019) Acrs can be further modified to change their selectivity and efficacy (Aschenbrenner et al., 2020; Osuna, 2019). AcrIIC1 gene that could be changed AcrIIC1X evolved from a Type II-C regulator which restricts class II-A SauCas9, depends on the structure of the Nme1Cas9 interacting contact with AcrIIC1 (Osuna, 2019). The suppressive efficacy of Cas9 activity could be fine-tuned by utilizing intentionally weakened Acr proteins to achieve an ideal-target editor and some off-target editor events are in a kinetic equilibrium (Aschenbrenner et al., 2020).

Other uses of Acr proteins;

Inhibitory Cas9 is also a beneficial strategy for creating vectors of viral infection with a "self-cleaving" chromosome. By design, a (helper-dependent adenovirus) HDAd vector for temporary SpyCas9 as well as guiding are encoded by Cas9 activation in specific receptors Cas9 manifestation in the intended recipient cells encodes SpyCas9 and breakage among the vector's genetic code later target cell modulation allowing transitory SpyCas9 expression and function (Palmer et al., 2019). Self-cleavage can

also occur throughout the viral generation, resulting in rearrangements in the genome that are impossible to develop and render virus production. SpyCas9 was 6inhi2 bitten from starting vector self-cleavage during viral generation using Acr proteins, considerably boosting yield (Palmer et al., 2019). Acr proteins could also be used to produce phage treatments as a replacement for antibiotics used to diagnose bacterial infections (Nobrega et al., 2015).

Cas9-pDBD fusion proteins improve the activity and precision of Cas9;

The pDBD linked to a PAM attenuated SpyCas9 provides an extended facility for high specificity and targeted range (Bolukbasi et al., 2015; Bolukbasi et al., 2018). Similarly, changes in some PID residues reduce Nme1Cas9's ability to bind to specific DNA (Amrani et al., in construction). We can use a simple variant (R1025A) and a dual variant (K1013A/R1025A) that demonstrate the more pronounced decrease of Nme1Cas9 intrinsically good DNA binding capacity with the definitive PAM identified as simple variant (SM) and dual variant (DM, as shown] (Amrani et al., in preparation). Due to Cas9 variations' decreased ability for PAM recognition, an extra DNA creating a component is required to engage and cleave target DNA, bolstering the nuclease's stronger affinity and precision. Nme1Cas9 mutants were linked to ZFPs, that are DNA-binding proteins (Amrani et al., 2018).

Inhibition of Cas9-pDBD proteins by Acr proteins;

The HEK293T molecules that had been co-transfected including vectors displaying the Acr protein, Nme1Cas9, and plasmid are use to target the NTS25 genomic region Type II-C Acr proteins could suppress Nme1Cas9-pDBD genome editing. T7E1 and TIDE were then utilised to determine genome editing efficacy. In the existence of Nme1Cas9WT, the appearance of Acrs significantly inhibited Nme1Cas9-induced mutations confirming our prior findings. They do not see any modification without any kind of Acr proteins, as indicated for Nme1Cas9SM because Nme1Cas9SM cannot attach to the target DNA without the pDBD. In the absence of Acrs, connecting the ZFP to Nme1Cas9SM (Zif268-Nme1Cas9SM) restores and even enhances Cas9 cleavage activity. In the Acr proteins, the increase in editing efficiency is nearly erased except for AcrIIC2Nme, all Type II-C Acrs reduced the breakage action of the forest-type and the ZFP-Nme1Cas9 merging protein. AcrIIC2Nme inhibits Nme1Cas9WT but not Nme1Cas9WT merged to a ZFP, which is surprising. This finding prompted us to see if AcrIIC2Nme can inhibit Nme1Cas9WT fused to a pDBD from off-targeting while not interfering with on-target site editing.

Cas9-Cas9 fusion proteins expand the utility of Cas9 genome editing

The pDBD adds a second step to the specific site licensing before to cross-linking in the chimaera between PAM-association suppressed Cas9 and pDBD, improving the targeted area, efficacy, and specificity (Amrani et al., 2018; Bolukbasi et al., 2015). However, generating operational pDBD mergers is more difficult than programming Cas9 with guide RNAs. In order to enable the adoption of Cas9's RNA-programmable binding platform over pDBD, antagonistic Cas9-Cas9 fused amino acids have been designed. Cas9-Cas9 fusions,(Bolukbasi et al., 2018) whether single- or dual-nuclease, have a wider targeting range and large specific surface area for genome editing. A couple of complementary mutations Cas9s have been coupled in particular to provide the specific lateral reductions used Cas9 (Bolukbasi et al., 2018).

CRISPR/Cas9 Therapeutic Application:

Most pharmaceutical industries and smaller biomedical begin are working on

Clustered regularly interspaced short palindromic therapeutics. CRISPR genome editing is expected to be quicker, lower expensive, and maybe more secure than the conventional gene therapy process. Autologous CRISPR gene cures, which use genome editing to correct an abnormality in a patient's own tissues, could eliminate the disapproval risks involved with provider transplantation therapies. CRISPR genome editing has special potential for disorders that can be treated by changing tissues which can be easily managed by an individual, according to researchers. Enabling for additional monitoring to ensure that no off-target genome modifications arise as a result of the editing procedure.

Reducing off-target effects:

When given to living cells at small doses and at the right time, AcrIIA4 was demonstrated to significantly minimize SpyCas9 off-target impacts while maintaining on-target SpyCas9-mediated gene editing (Shin et al., 2017). SpyCas9-sgRNA activity is vir Controlling tually completely reduced when AcrIIA4 has produced 24 hours ago SpyCas9 RNP stimulation. When AcrIIA4 is introduced to cells 6 hours through the SpyCas9-sgRNA compound is formed, genetic manipulation is only reduced by roughly fifty percent (Shin et al., 2017).

The activity of SpyCas9 RNPs can be monitored rather than putting into consideration several of these parameters, according to Aschenbrenner and coauthors simply combining SpyCas9 to mutant (weaker) forms of AcrIIA4 (Aschenbrenner et al., 2020). SpyCas9 will have too much time to alter – only – the center of intrest (higher affinity) Before being deactivated in this way. In theory, this technique reduces off-target effects significantly while having no observable negative effects on ontarget editing. However, with different sgRNAs, the method's performance varies

Controlling gene drive

CRISPR-Cas9 is a dependable mechanism for achieving gene drive, or the spread of a specific gene throughout a population (Hammond et al., 2016). Basgall and co-authors (Basgall et al., 2018) used AcrIIA2 and AcrIIA4 to completely abolish gene drive in *S. cerevisiae*. They also created a gene drive mechanism that can be induced in which SpyCas9 and AcrIIA2/A4 were controlled by two distinct regulated promoters, GAL1 and MET25, roughly. MET25 promoter is blocked by methionine, but the GAL1 promoter is stimulated by galactose. SpyCas9 was produced by yeast cells starting to grow in a medium containing both galactose and methionine which when bound to a suitably designed sgRNA, resulted in gene drive. Anti CRISPR amino acids were created and the gene drive was turned off when yeast cells were grown in a material that lacked methionine. Importantly, this research included a thorough examination of both AcrIIA2 and AcrII4, to determine which metabolites are critical for the suppressive action of the two proteins. An impartial alanine scan revealed how distinct mutational sites influenced Acractivity to vary degrees. 22 AcrIIA2 and 19 AcrIIA4 mutations were created: 8 completely shed their disruptive action (6 AcrIIA2 and 2 AcrIIA4 variations), and five people are still employed but at a lower grade than wild-type proteins (two AcrIIA2 and three AcrIIA4 mutants). All of these mutations as well as those created through simple and multiple mutations on AcrIIA4 were studied when it comes to the gene drive. This experiment proved that Acrmutations can be used to fine-tune gene drive effects.

Nme1Cas9-guided programmable RNA editing;

Nme1Cas9 is unique among Cas9 family members in that it may target single-stranded RNA (ssRNA) without the presence of a PAM pattern. Without the need for any RNA-

PAM, ssRNA-crRNA complementary base-pairing is used to recognize and select the target ssRNA (or rPAM). As a result, Nme1Cas9 may execute Unique to the location ssRNA cleavage without causing damage to the dsDNA. AcrIIC1-C3 also inhibits ssRNA cleavage (Rousseau et al., 2018). Using Nme1Cas9 and AcrIIC1-C3, this discovery provides a novel platform for manipulating ssRNA (Rousseau et al., 2018).

Summary

In certain situations, the CRISPR-Cas adaptive immune defense organizations seen in bacteria and Archaea may give discrete sequence defense in contradiction to invading DNA or RNA. Acr genes produce proteins that attach to the unit facilitating the defense process and so counteract CRISPR-Cas immune mechanisms in bacteria, despite the emergence of defense mechanisms in bacterial hosts. In the future, more Acrs are expected to be identified, and their activities and responsibilities should be extensively researched. Acrs increased understanding might contribute to their extensive use as efficient controllers of CRISPR-Cas system-associated genome editing, which could aid mankind in overcoming diseases. As CRISPR becomes more commonly employed in human medicine, techniques for hindering Cas enzymes will become increasingly useful. In the upcoming days, blockers might be applied to lower CRISPR activity in non-target tissues during gene therapy, added proactively to diminish off-target action, or even employed in an emergency when CRISPR has been misused or has generated poor results.

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Scientific Ethics Declaration

The authors say that they are responsible for the scientific, moral, and legal aspects of this chapter, which is published in Current Studies in Health and Life Sciences 2023.

Conflict of Interest

The authors declare no conflict of interest.

References

1. Brito, I.L., *Examining horizontal gene transfer in microbial communities*. Nature Reviews Microbiology, 2021. **19**(7): p. 442-453.
2. Soucy, S.M., J. Huang, and J.P. Gogarten, *Horizontal gene transfer: building the web of life*. Nature Reviews Genetics, 2015. **16**(8): p. 472-482.
3. Mejdani, M., *Acr and CRISPR-Cas: Characterization and Biotechnology*. 2019, University of Toronto (Canada).
4. Tang, X.-D., et al., *Methods for enhancing clustered regularly interspaced short palindromic repeats/Cas9-mediated homology-directed repair efficiency*. Frontiers in genetics, 2019. **10**: p. 551.
5. Davidson, A.R., et al., *Acr: protein inhibitors of CRISPR-Cas systems*. Annual review of biochemistry, 2020. **89**: p. 309-332.
6. Hampton, H.G., B.N. Watson, and P.C. Fineran, *The arms race between bacteria and their phage foes*. Nature, 2020. **577**(7790): p. 327-336.

7. Jia, N. and D.J. Patel, *Structure-based functional mechanisms and biotechnology applications of Acr proteins*. Nature Reviews Molecular Cell Biology, 2021. **22**(8): p. 563-579.
8. Eaglesham, J.B. and P.J. Kranzusch, *Conserved strategies for pathogen evasion of cGAS–STING immunity*. Current opinion in immunology, 2020. **66**: p. 27-34.
9. Cheng, Z., et al., *The interactions between cGAS-STING pathway and pathogens*. Signal transduction and targeted therapy, 2020. **5**(1): p. 1-15.
10. Deveau, H., et al., *Phage response to CRISPR-encoded resistance in Streptococcus thermophilus*. Journal of bacteriology, 2008. **190**(4): p. 1390-1400.
11. Mendoza, S.D., et al., *A bacteriophage nucleus-like compartment shields DNA from CRISPR nucleases*. Nature, 2020. **577**(7789): p. 244-248.
12. Malone, L.M., et al., *A jumbo phage that forms a nucleus-like structure evades CRISPR–Cas DNA targeting but is vulnerable to type III RNA-based immunity*. Nature microbiology, 2020. **5**(1): p. 48-55.
13. Samson, J.E., et al., *Revenge of the phages: defeating bacterial defences*. Nature Reviews Microbiology, 2013. **11**(10): p. 675-687.
14. Bondy-Denomy, J., et al., *Bacteriophage genes that inactivate the CRISPR/Cas bacterial immune system*. Nature, 2013. **493**(7432): p. 429-432.
15. Makarova, K.S., et al., *Evolutionary classification of CRISPR–Cas systems: a burst of class 2 and derived variants*. Nature Reviews Microbiology, 2020. **18**(2): p. 67-83.
16. Makarova, K.S., et al., *An updated evolutionary classification of CRISPR–Cas systems*. Nature Reviews Microbiology, 2015. **13**(11): p. 722-736.
17. Van Der Oost, J., et al., *Unravelling the structural and mechanistic basis of CRISPR–Cas systems*. Nature Reviews Microbiology, 2014. **12**(7): p. 479-492.
18. Marraffini, L.A., *CRISPR-Cas immunity in prokaryotes*. Nature, 2015. **526**(7571): p. 55-61.
19. Andersson, A.F. and J.F. Banfield, *Virus population dynamics and acquired virus resistance in natural microbial communities*. Science, 2008. **320**(5879): p. 1047-1050.
20. Hwang, S. and K.L. Maxwell, *Meet the Acr: widespread protein inhibitors of CRISPR-Cas systems*. The CRISPR journal, 2019. **2**(1): p. 23-30.
21. Marino, N.D., et al., *Acr protein applications: natural brakes for CRISPR-Cas technologies*. Nature methods, 2020. **17**(5): p. 471-479.
22. Jinek, M., et al., *A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity*. science, 2012. **337**(6096): p. 816-821.
23. Mali, P., et al., *RNA-guided human genome engineering via Cas9*. Science, 2013. **339**(6121): p. 823-826.
24. Cong, L., et al., *Multiplex genome engineering using CRISPR/Cas systems*. Science, 2013. **339**(6121): p. 819-823.
25. Qi, L.S., et al., *Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression*. Cell, 2013. **152**(5): p. 1173-1183.
26. Hsu, P.D., E.S. Lander, and F. Zhang, *Development and applications of CRISPR-Cas9 for genome engineering*. Cell, 2014. **157**(6): p. 1262-1278.
27. Adli, M., *The CRISPR tool kit for genome editing and beyond*. Nature communications, 2018. **9**(1): p. 1-13.

28. Kempton, H.R. and L.S. Qi, *When genome editing goes off-target*. Science, 2019. **364**(6437): p. 234-236.
29. Li, C., et al., *HDAd5/35++ adenovirus vector expressing Acrpeptides decreases CRISPR/Cas9 toxicity in human hematopoietic stem cells*. Molecular Therapy-Methods & Clinical Development, 2018. **9**: p. 390-401.
30. Li, C., et al., *In vivo HSC gene therapy using a bi-modular HDAd5/35++ vector cures sickle cell disease in a mouse model*. Molecular Therapy, 2021. **29**(2): p. 822-837.
31. Pawluk, A., et al., *Disabling a type I E CRISPR-Cas nuclease with a bacteriophage-encoded Acr protein*. MBio, 2017. **8**(6): p. e01751-17.
32. Meaden, S., et al., *Phage gene expression and host responses lead to infection-dependent costs of CRISPR immunity*. The ISME journal, 2021. **15**(2): p. 534-544.
33. Pawluk, A., A.R. Davidson, and K.L. Maxwell, *Anti-CRISPR: discovery, mechanism and function*. Nature Reviews Microbiology, 2018. **16**(1): p. 12-17.
34. Touchon, M. and E.P. Rocha, *The small, slow and specialized CRISPR and Acrof Escherichia and Salmonella*. PloS one, 2010. **5**(6): p. e11126.
35. Bondy-Denomy, J., et al., *Multiple mechanisms for CRISPR-Cas inhibition by Acr proteins*. Nature, 2015. **526**(7571): p. 136-139.
36. Borges, A.L., A.R. Davidson, and J. Bondy-Denomy, *The discovery, mechanisms, and evolutionary impact of Acr*. Annual review of virology, 2017. **4**: p. 37-59.
37. Stanley, S.Y., et al., *Anti-CRISPR-associated proteins are crucial repressors of Acrtranscription*. Cell, 2019. **178**(6): p. 1452-1464. e13.
38. Peng, X., et al., *Acr proteins in archaea*. Trends in Microbiology, 2020. **28**(11): p. 913-921.
39. Liu, Q., H. Zhang, and X. Huang, *Anti-CRISPR proteins targeting the CRISPR-Cas system enrich the toolkit for genetic engineering*. The FEBS journal, 2020. **287**(4): p. 626-644.
40. e Pacheco, B.I.C., *Physiological and genomic characterization of six virulent bacteriophages of Shiga toxin-producing Escherichia coli O157: H7 for biocontrol and detection applications*. 2019.
41. Derrien, T., et al., *The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression*. Genome research, 2012. **22**(9): p. 1775-1789.
42. Shehreen, S., *The impact of Acr on Horizontal Gene Transfer (HGT) and their regulation in mobile genetic elements*. 2021, University of Otago.
43. Yin, Y., B. Yang, and S. Entwistle, *Bioinformatics identification of Acrloci by using homology, guilt-by-association, and CRISPR self-targeting spacer approaches*. Msystems, 2019. **4**(5): p. e00455-19.
44. Walker, F.C., *CRISPR Regulation and RNA Biogenesis in Staphylococcus epidermidis*. 2017: The University of Alabama.
45. Pawluk, A.I.E., *Prevalence, Diversity, Impact, and Applications of CRISPR-Cas Inhibitors*. 2016, University of Toronto (Canada).
46. Zhu, Y., et al., *Diverse mechanisms of CRISPR-Cas9 inhibition by type IIC Acr proteins*. Molecular cell, 2019. **74**(2): p. 296-309. e7.
47. Porto, E.M., et al., *Base editing: advances and therapeutic opportunities*. Nature Reviews Drug Discovery, 2020. **19**(12): p. 839-859.
48. Bondy-Denomy, J., *Protein inhibitors of CRISPR-Cas9*. ACS chemical biology, 2018. **13**(2): p. 417-423.

49. Yaung, S.J., K.M. Esvelt, and G.M. Church, *CRISPR/Cas9-mediated phage resistance is not impeded by the DNA modifications of phage T4*. PloS one, 2014. **9**(6): p. e98811.
50. Reinshagen, C., et al., *CRISPR-enhanced engineering of therapy-sensitive cancer cells for self-targeting of primary and metastatic tumors*. Science translational medicine, 2018. **10**(449): p. eaao3240.
51. Pinilla-Redondo, R., et al., *Discovery of multiple Acr highlights anti-defense gene clustering in mobile genetic elements*. Nature communications, 2020. **11**(1): p. 1-11.
52. Hille, F. and E. Charpentier, *CRISPR-Cas: biology, mechanisms and relevance*. Philosophical transactions of the royal society B: biological sciences, 2016. **371**(1707): p. 20150496.
53. Osuna, B.A., et al., *Listeria phages induce Cas9 degradation to protect lysogenic genomes*. Cell host & microbe, 2020. **28**(1): p. 31-40. e9.
54. Zhang, Y. and M.A. Marchisio, *Type II Acr proteins as a new tool for synthetic biology*. RNA biology, 2021. **18**(8): p. 1085-1098.
55. Leon, L.M., *CRISPR-Cas3: Studying the molecular interactions that drive adaptation & engineering novel bacterial editing tools*. 2021: University of California, San Francisco.
56. Yi, H., et al., *AcrFinder: genome mining Acroperons in prokaryotes and their viruses*. Nucleic acids research, 2020. **48**(W1): p. W358-W365.
57. Birkholz, N., et al., *The autoregulator Aca2 mediates Acrrepression*. Nucleic acids research, 2019. **47**(18): p. 9658-9665.
58. Forsberg, K.J., et al., *The novel AcrAcrIIA22 relieves DNA torsion in target plasmids and impairs SpyCas9 activity*. PLoS biology, 2021. **19**(10): p. e3001428.
59. Thavalingam, A., *The Functional Characterization of AcrAcrIIC2 Nme*. 2019, University of Toronto (Canada).
60. Pawluk, A., et al., *Naturally occurring off-switches for CRISPR-Cas9*. Cell, 2016. **167**(7): p. 1829-1838. e9.
61. Webber, B.L., S. Raghu, and O.R. Edwards, *Opinion: Is CRISPR-based gene drive a biocontrol silver bullet or global conservation threat?* Proceedings of the National Academy of Sciences, 2015. **112**(34): p. 10565-10567.
62. Scheben, A., et al., *Towards CRISPR/Cas crops—bringing together genomics and genome editing*. New Phytologist, 2017. **216**(3): p. 682-698.
63. Huang, C.-H., K.-C. Lee, and J.A. Doudna, *Applications of CRISPR-Cas enzymes in cancer therapeutics and detection*. Trends in cancer, 2018. **4**(7): p. 499-512.
64. Lino, C.A., et al., *Delivering CRISPR: a review of the challenges and approaches*. Drug delivery, 2018. **25**(1): p. 1234-1257.
65. Karimian, A., et al., *CRISPR/Cas9 technology as a potent molecular tool for gene therapy*. Journal of cellular physiology, 2019. **234**(8): p. 12267-12277.
66. Babačić, H., et al., *CRISPR-cas gene-editing as plausible treatment of neuromuscular and nucleotide-repeat-expansion diseases: A systematic review*. PloS one, 2019. **14**(2): p. e0212198.
67. Yasaka, M., et al., *Correction of INR by prothrombin complex concentrate and vitamin K in patients with warfarin related hemorrhagic complication*. Thrombosis research, 2002. **108**(1): p. 25-30.
68. CR, L., *The neutralization of heparin by protamine*. Surgery, 1948. **24**(1): p. 97-99.

69. Hammond, A., et al., *A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector Anopheles gambiae*. Nature biotechnology, 2016. **34**(1): p. 78-83.
70. Zhang, F., G. Song, and Y. Tian, *Anti-CRISPRs: The natural inhibitors for CRISPR-Cas systems*. Animal models and experimental medicine, 2019. **2**(2): p. 69-75.
71. Barboni, M., et al., *Early formation of the Moon 4.51 billion years ago*. Science advances, 2017. **3**(1): p. e1602365.
72. Naeem, M., et al., *Latest developed strategies to minimize the off-target effects in CRISPR-Cas-mediated genome editing*. Cells, 2020. **9**(7): p. 1608.
73. Zuccaro, M.V., et al., *Allele-specific chromosome removal after Cas9 cleavage in human embryos*. Cell, 2020. **183**(6): p. 1650-1664. e15.
74. Shen, C.-C., et al., *Synthetic switch to minimize CRISPR off-target effects by self-restricting Cas9 transcription and translation*. Nucleic acids research, 2019. **47**(3): p. e13-e13.
75. Shin, J., et al., *Disabling Cas9 by an AcrDNA mimic*. Science advances, 2017. **3**(7): p. e1701620.
76. Kosicki, M., K. Tomberg, and A. Bradley, *Repair of double-strand breaks induced by CRISPR-Cas9 leads to large deletions and complex rearrangements*. Nature biotechnology, 2018. **36**(8): p. 765-771.
77. Meltzer, H., et al., *Tissue-specific (ts) CRISPR as an efficient strategy for in vivo screening in Drosophila*. Nature communications, 2019. **10**(1): p. 1-9.
78. Wang, X.-W., et al., *A microRNA-inducible CRISPR-Cas9 platform serves as a microRNA sensor and cell-type-specific genome regulation tool*. Nature cell biology, 2019. **21**(4): p. 522-530.
79. Wei, T., et al., *Systemic nanoparticle delivery of CRISPR-Cas9 ribonucleoproteins for effective tissue specific genome editing*. Nature communications, 2020. **11**(1): p. 1-12.
80. Pawluk, A., et al., *A new group of phage Acr genes inhibits the type IE CRISPR-Cas system of Pseudomonas aeruginosa*. MBio, 2014. **5**(2): p. e00896-14.
81. Rauch, B.J., et al., *Inhibition of CRISPR-Cas9 with bacteriophage proteins*. Cell, 2017. **168**(1-2): p. 150-158. e10.
82. Hynes, A.P., et al., *An Acr from a virulent streptococcal phage inhibits Streptococcus pyogenes Cas9*. Nature microbiology, 2017. **2**(10): p. 1374-1380.
83. Garcia, B., et al., *AcrAcrIIA5 potently inhibits all Cas9 homologs used for genome editing*. Cell reports, 2019. **29**(7): p. 1739-1746. e5.
84. Song, G., et al., *AcrIIA5 inhibits a broad range of Cas9 orthologs by preventing DNA target cleavage*. Cell reports, 2019. **29**(9): p. 2579-2589. e4.
85. Watters, K.E., et al., *Potent CRISPR-Cas9 inhibitors from Staphylococcus genomes*. Proceedings of the National Academy of Sciences, 2020. **117**(12): p. 6531-6539.
86. Watters, K.E., et al., *Systematic discovery of natural CRISPR-Cas12a inhibitors*. Science, 2018. **362**(6411): p. 236-239.
87. Chowdhury, S., et al., *Structure reveals mechanisms of viral suppressors that intercept a CRISPR RNA-guided surveillance complex*. Cell, 2017. **169**(1): p. 47-57. e11.
88. Maxwell, K.L., et al., *The solution structure of an Acr protein*. Nature communications, 2016. **7**(1): p. 1-5.

89. Guo, T.W., et al., *Cryo-EM structures reveal mechanism and inhibition of DNA targeting by a CRISPR-Cas surveillance complex*. Cell, 2017. **171**(2): p. 414-426. e12.
90. Wang, X., et al., *Structural basis of Cas3 inhibition by the bacteriophage protein AcrF3*. Nature structural & molecular biology, 2016. **23**(9): p. 868-870.
91. Ka, D., et al., *Crystal structure of an Acr protein, AcrIIA1*. Nucleic acids research, 2018. **46**(1): p. 485-492.
92. Dong, D., et al., *Structural basis of CRISPR–SpyCas9 inhibition by an Acr protein*. Nature, 2017. **546**(7658): p. 436-439.
93. Harrington, L.B., et al., *A broad-spectrum inhibitor of CRISPR-Cas9*. Cell, 2017. **170**(6): p. 1224-1233. e15.
94. Mallon, J. and S. Bailey, *A molecular arms race: New insights into Acr mechanisms*. Nature structural & molecular biology, 2016. **23**(9): p. 765-766.
95. Wang, J., et al., *A CRISPR evolutionary arms race: structural insights into viral anti-CRISPR/Cas responses*. Cell research, 2016. **26**(10): p. 1165-1168.
96. Holm, L. and P.i. Rosenström, *Dali server: conservation mapping in 3D*. Nucleic acids research, 2010. **38**(suppl_2): p. W545-W549.
97. Nakamura, M., et al., *Anti-CRISPR-mediated control of gene editing and synthetic circuits in eukaryotic cells*. Nature communications, 2019. **10**(1): p. 1-11.
98. Basgall, E.M., et al., *Gene drive inhibition by the Acr proteins AcrIIA2 and AcrIIA4 in Saccharomyces cerevisiae*. Microbiology, 2018. **164**(4): p. 464.
99. Yang, H. and D.J. Patel, *Inhibition mechanism of an Acr suppressor AcrIIA4 targeting SpyCas9*. Molecular cell, 2017. **67**(1): p. 117-127. e5.
100. Jinek, M., et al., *Structures of Cas9 endonucleases reveal RNA-mediated conformational activation*. Science, 2014. **343**(6176): p. 1247-997.
101. Jiang, F., et al., *A Cas9–guide RNA complex preorganized for target DNA recognition*. Science, 2015. **348**(6242): p. 1477-1481.
102. Liu, L., et al., *Phage AcrIIA2 DNA mimicry: structural basis of the CRISPR and Acr arms race*. Molecular cell, 2019. **73**(3): p. 611-620. e3.
103. Hynes, A.P., et al., *Widespread Acr proteins in virulent bacteriophages inhibit a range of Cas9 proteins*. Nature communications, 2018. **9**(1): p. 1-10.
104. An, S.Y., et al., *Intrinsic disorder is essential for Cas9 inhibition of AcrIIA5*. Nucleic acids research, 2020. **48**(13): p. 7584-7594.
105. Fuchsbauer, O., et al., *Cas9 allosteric inhibition by the Acr protein AcrIIA6*. Molecular cell, 2019. **76**(6): p. 922-937. e7.
106. Uribe, R.V., et al., *Discovery and characterization of Cas9 inhibitors disseminated across seven bacterial phyla*. Cell host & microbe, 2019. **25**(2): p. 233-241. e5.
107. Forsberg, K.J., et al., *Functional metagenomics-guided discovery of potent Cas9 inhibitors in the human microbiome*. eLife, 2019. **8**: p. e46540.
108. Pawluk, A., et al., *Inactivation of CRISPR-Cas systems by Acr proteins in diverse bacterial species*. Nature microbiology, 2016. **1**(8): p. 1-6.
109. Gussow, A.B., et al., *Machine-learning approach expands the repertoire of Acr protein families*. Nature communications, 2020. **11**(1): p. 1-12.
110. Eitzinger, S., et al., *Machine learning predicts new Acr proteins*. Nucleic acids research, 2020. **48**(9): p. 4698-4708.
111. Lee, J., et al., *Potent Cas9 inhibition in bacterial and human cells by AcrIIC4 and AcrIIC5 Acr proteins*. MBio, 2018. **9**(6): p. e02321-18.

112. Edraki, A., et al., *A compact, high-accuracy Cas9 with a dinucleotide PAM for in vivo genome editing*. Molecular cell, 2019. **73**(4): p. 714-726. e4.
113. Sun, W., et al., *Structures of Neisseria meningitidis Cas9 complexes in catalytically poised and anti-CRISPR-inhibited states*. Molecular cell, 2019. **76**(6): p. 938-952. e5.
114. Sternberg, S.H., et al., *Conformational control of DNA target cleavage by CRISPR–Cas9*. Nature, 2015. **527**(7576): p. 110-113.
115. Kim, Y., et al., *Anti-CRISPR AcrIIC3 discriminates between Cas9 orthologs via targeting the variable surface of the HNH nuclease domain*. The FEBS journal, 2019. **286**(23): p. 4661-4674.
116. Thavalingam, A., et al., *Inhibition of CRISPR-Cas9 ribonucleoprotein complex assembly by AcrAcrIIC2*. Nature communications, 2019. **10**(1): p. 1-11.
117. Kahne, D. and W.C. Still, *Hydrolysis of a peptide bond in neutral water*. Journal of the American Chemical Society, 1988. **110**(22): p. 7529-7534.
118. Meijer, A.J. and P. Codogno, *Regulation and role of autophagy in mammalian cells*. The international journal of biochemistry & cell biology, 2004. **36**(12): p. 2445-2462.
119. Glickman, M.H. and A. Ciechanover, *The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction*. Physiological reviews, 2002.
120. Matsumoto, D., H. Tamamura, and W. Nomura, *A cell cycle-dependent CRISPR-Cas9 activation system based on an Acr protein shows improved genome editing accuracy*. Communications biology, 2020. **3**(1): p. 1-10.
121. Nateghi Rostami, M., *CRISPR/Cas9 gene drive technology to control transmission of vector-borne parasitic infections*. Parasite immunology, 2020. **42**(9): p. e12762.
122. Bubeck, F., et al., *Engineered Acr proteins for optogenetic control of CRISPR–Cas9*. Nature methods, 2018. **15**(11): p. 924-927.
123. Hoffmann, M.D., et al., *Cell-specific CRISPR–Cas9 activation by microRNA-dependent expression of Acr proteins*. Nucleic acids research, 2019. **47**(13): p. e75-e75.
124. Mathony, J., et al., *Computational design of Acr proteins with improved inhibition potency*. Nature Chemical Biology, 2020. **16**(7): p. 725-730.
125. Aschenbrenner, S., et al., *Coupling Cas9 to artificial inhibitory domains enhances CRISPR-Cas9 target specificity*. Science advances, 2020. **6**(6): p. eaay0187.
126. Osuna, B., *Surveillance and Defense Mechanisms in Microbes*. 2019: University of California, San Francisco.
127. Palmer, D.J., D.L. Turner, and P. Ng, *Production of CRISPR/Cas9-mediated self-cleaving helper-dependent adenoviruses*. Molecular Therapy-Methods & Clinical Development, 2019. **13**: p. 432-439.
128. Nobrega, F.L., et al., *Revisiting phage therapy: new applications for old resources*. Trends in microbiology, 2015. **23**(4): p. 185-191.
129. Bolukbasi, M.F., et al., *DNA-binding-domain fusions enhance the targeting range and precision of Cas9*. Nature methods, 2015. **12**(12): p. 1150-1156.
130. Bolukbasi, M.F., et al., *Orthogonal Cas9–Cas9 chimeras provide a versatile platform for genome editing*. Nature communications, 2018. **9**(1): p. 1-12.
131. Amrani, N., et al., *NmeCas9 is an intrinsically high-fidelity genome-editing platform*. Genome Biology, 2018. **19**(1): p. 1-25.

132. Rousseau, B.A., et al., *Programmable RNA cleavage and recognition by a natural CRISPR-Cas9 system from Neisseria meningitidis*. Molecular cell, 2018. **69**(5): p. 906-914. e4.

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