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Keeping CRISPR in check: diverse mechanisms of phage-encoded anti-CRISPRS

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One sentence summary: In this review, we discuss our current understanding of phage-encoded inhibitors that block prokaryotic CRISPR-Cas systems, their mechanisms, structures and applications.

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ABSTRACT

CRISPR-Cas represents the only adaptive immune system of prokaryotes known to date. These immune systems are widespread among bacteria and archaea, and provide protection against invasion of mobile genetic elements, such as bacteriophages and plasmids. As a result of the arms-race between phages and their prokaryotic hosts, phages have evolved inhibitors known as anti-CRISPR (Acr) proteins to evade CRISPR immunity. In the recent years, several Acr proteins have been described in both temperate and virulent phages targeting diverse CRISPR-Cas systems. Here, we describe the strategies of Acr discovery and the multiple molecular mechanisms by which these proteins operate to inhibit CRISPR immunity. We discuss the biological relevance of Acr proteins and speculate on the implications of their activity for the development of improved CRISPR-based research and biotechnological tools.

Keywords: CRISPR-cas; phage; genome editing; anti-CRISPR

INTRODUCTION

Viruses are ubiquitous entities co-existing with cellular life forms, present in almost all explored environments (Koonin and Dolja 2013). Viruses that infect bacteria (bacteriophages or phages) are the most abundant biological entities on the planet with population numbers in the order of $10^{31}$ (Suttle 2005; Koonin and Dolja 2013; Guemes et al. 2016). The ability of phages to easily manoeuvre between different biomes, operating as vehicles of horizontal gene transfer (HGT), makes them major agents of evolution (Sano et al. 2004). Bacteriophages are classified based on their life-cycle into virulent and temperate. Virulent phages rely exclusively on productive infection cycles for propagation, which ultimately kills the host for the release of new viral particles that can engage in another round of infection. Temperate phages have the choice to multiply in their host cells leading to cell lysis or to integrate their phage genome into the bacterial chromosome as a prophage. Prophages are propagated passively by the replication machinery of the bacterial cell (Gandon 2016).
As a response to the constant threat of phage infection, a diverse arsenal of defence mechanisms has evolved in bacterial hosts. Because phages evolve rapidly to counter these immune systems (Drake et al. 1998), the hosts need to constantly evolve new means of self-protection, leading to a perennial arms-race between hosts and their phages (Forterre and Prangishvili 2009). The defence systems evolved by bacteria provide both innate and adaptive immunity against phage infection. Innate immunity systems interfere at different levels of the phage’s infection cycle via receptor masking, superinfection exclusion (Sie), restriction-modification (R-M), bacteriophage exclusion (BREX), toxin–antitoxin (TA) modules, abortive infection (Abi), prokaryotic Argonautes (pAgos), production of anti-phage chemicals and defence island system associated with restriction–modification (DISARM) systems (Chopin, Chopin and Bidnenko 2005; Makarova et al. 2011; Makarova, Wolf and Koonin 2013; Samson et al. 2013; Goldfarb et al. 2015; Kronheim et al. 2018), whereas other remain to be characterized (Doron et al. 2018). Adaptive and heritable immunity is provided by Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)–CRISPR-associated (Cas) systems (Barrangou et al. 2007), which work as a fascinating complementation to the innate defence strategies.

Diverse variants of the CRISPR-Cas defence system are present in most of the sequenced genomes of archaea and half of those of bacteria (Makarova et al. 2013). A CRISPR-Cas locus typically consists of a CRISPR array and an operon of CRISPR-associated (cas) genes. The CRISPR array is composed of a series of short, partially palindromic and direct repetitive sequences (repeats) interspaced by variable sequences (spacers), originating from phage genomes or other invading mobile genetic elements (MGE), such as (conjugative) plasmids (Bolotin et al. 2005; Mojica et al. 2005; Poursel, Salvignol and Vergnaud 2005; Shmakov et al. 2017). The cas genes encode for the Cas proteins, which are necessary for the generation of new spacers or are involved in the targeting of the MGE, as explained below. Collectively, these two elements of CRISPR-Cas systems mediate sequence-specific immunity against invasive MGEs (Brouns et al. 2008; Marraffini and Sontheimer 2008; Hale et al. 2009; Garneau et al. 2010).

The continuous arms-race between propharyotic hosts and their cognate MGEs is speculated to be responsible for the rapid evolution of highly diverse CRISPR-Cas systems. The current CRISPR-Cas classification scheme distinguishes two broad classes based on the protein composition of the effector Cas complex. Class 1 systems (types I, III and IV) use multi-subunit Cas protein complexes for the recognition of targeted nucleic acids, while the less common class 2 systems (types II, V and VI) employ a single multi-domain effector protein complex that performs target recognition and cleavage. These classes are further subdivided into a total of six CRISPR types with 25 subtypes (Koonin, Makarova and Zhang 2017).

Despite substantial structural and functional diversity, all CRISPR-Cas systems mediate immunity through three distinct steps: adaptation, expression and interference (Mohanraju et al. 2016). During adaptation, short DNA fragments (known as protospacers) are acquired from invading MGEs and subsequently processed and inserted as spacers into the CRISPR locus, typically by the Cas1–Cas2 complex (Jackson et al. 2017). Next, during expression, the CRISPR array is transcribed as a long precursor CRISPR RNA (pre-crRNA) and the Cas proteins are expressed. The pre-crRNA is then processed within repeat regions to yield mature CRISPR RNAs (crRNAs) by dedicated Cas proteins and/or host factors (Brouns et al. 2008; Hale et al. 2008; Haurwitz et al. 2010; Deltcheva et al. 2011). The crRNAs are packaged with one or more Cas proteins into effector Cas complexes that scrutinise the microbial cell for potential invasion. Finally, during interference, the Cas complexes recognize complementary target sequences of invading MGEs by Watson–Crick base-pairing. Upon binding to a cognate target sequence, the complex either recruits a nuclease or stimulates its intrinsic nucleolytic activity to neutralize the invader (Brouns et al. 2008; Garneau et al. 2010; Westra et al. 2012). Type I, II and V CRISPR-Cas systems target DNA and rely on a short stretch (2 to 7 nucleotides) of conserved nucleotides adjacent to the protospacer, known as the protospacer-adjacent motif (PAM), for spacer selection during adaptation and target identification during interference (Mojica et al. 2005; Marraffini and Sontheimer 2008). The PAM allows for self/nonself discrimination, as its absence in the CRISPR array prevents autoimmunity and self-cleavage.

In response to the microbial antiviral defence mechanisms, phages have evolved numerous mechanisms to overcome prokaryotic immunity. Phage evasion from propharyotic CRISPR-Cas systems was first found to rely on mutational drifts, predominantly occurring in regions that require perfect complementarity between the crRNA and the protospacer (so-called seed region) for interference, or in the PAM sequences (Deveau et al. 2008; Sun et al. 2013; Bondy-Denomy et al. 2015). Depending on the location of the mismatch (between the crRNA and the protospacer), a single mutation can be sufficient to abolish CRISPR-Cas immunity (Deveau et al. 2008; Semenova et al. 2011). Deletion of the protospacer sequence and/or the PAM have also been shown to provide an effective way for phages to escape CRISPR-Cas targeting, despite the potential of imposing a fitness cost (Deveau et al. 2008). Similar to the evasion strategy from R-M systems, phages can also modify their bases with hydroxymethylcytosine (HMC) and its bulkier glycosylated form to reduce target binding affinity and thereby protect from CRISPR-mediated targeting by both type I and type II systems (Bryson et al. 2015; Vlot et al. 2018), whereas other modifications do not disturb Cas9 recognition (Young, Esvelt and Church 2014). Finally, some phages encode their own CRISPR locus that targets host antiviral genomic regions, such as chromosomal (defence) islands (Seed et al. 2013).

The first examples of phage-encoded anti-CRISPR (Acr) proteins were found in class 1 type I-F and I-E systems of Pseudomonas aeruginosa (Bondy-Denomy et al. 2013; Pawluk et al. 2014). Acr proteins have distinct sequences (Tables 1 and 2), structures (Maxwell et al. 2016; Wang et al. 2016a; Harrington et al. 2017) and mechanisms (Bondy-Denomy et al. 2015) and they provide phages with a direct and specific means to inhibit targeting by the CRISPR-Cas system. To date, 45 unique families of Acr proteins have been discovered, and categorized into class 1 (Table 1) and class 2 (Table 2) CRISPR-Cas inhibitors. These highly diverse and small (typically 50–330 amino acids) proteins do not share much sequence or protein domain similarity to each other or to any protein of known function (Marino et al. 2018).

Here, we explore the biological relevance and detail the recent insights into the molecular mechanisms and structures of anti-CRISPR proteins. We also address the development of anti-CRISPRs as ‘off-switches’ for genome editing and discuss the impact of their use in other biotechnological applications.

**BIOLOGICAL RELEVANCE OF ANTI-CRISPR PROTEINS**

The emergence of widespread, specialized and highly diverse phage-encoded proteins that thwart CRISPR-Cas immunity, suggests that Acr proteins play an important role in phage
### Table 1. Class 1 anti-CRISPR protein families.

| Family | Size (aa) | Origin (characterized homolog) | Method of discovery | Accession number | CRISPR-Cas type inhibited [organism] | Mode-of-action | Structure | PDB code | References |
|--------|-----------|--------------------------------|---------------------|------------------|--------------------------------------|----------------|-----------|----------|------------|
| AcrIC1 | 190       | Moraxella bovoculi prophage    | Self-targeting, Guilt-by-association [acrF11] | AKG19229.1       | I-C [Mbo]                            | Binds as a dimer to the Cas10d, mimicking DNA (blocks DNA binding) | Compact dimeric αβ-sandwich; each monomer 5-stranded antiparallel β-sheet + 2 α-helices at one side of the β-sheet | 6EXP     | (Marino et al. 2018) |
| AcrID1 | 98        | Sulfolobus islandicus rudivirus 3 | Functional assays   | YP_009272954.1   | I-D [Sis]                            | Binds as a dimer to the Cas10d, mimicking DNA (blocks DNA binding) | Compact dimeric αβ-sandwich; each monomer 5-stranded antiparallel β-sheet + 2 α-helices at one side of the β-sheet | 6EXP     | (He et al. 2018)     |
| AcrIE1 | 100       | Pseudomonas aeruginosa phage JBD5 | Functional assays   | YP_007392738.1   | I-E [Pae]                            | Binds as a dimer to the Cas3 (blocks DNA cleavage)       | Elongated dimeric structure; each monomer 1 antiparallel β-sheet + 3 α-helices | 6ARZ, 6AS4 | (Pawluk et al. 2014), (Pawluk et al. 2017) |
| AcrIE2 | 84        | P. aeruginosa phage JBD88a     | Functional assays   | YP_007392439.1   | I-E [Pae]                            | —                          | —                     | —        | —          | (Pawluk et al. 2014) |
| AcrIE3 | 68        | P. aeruginosa phage DMS3       | Functional assays   | YP_950454.1      | I-E [Pae]                            | —                          | —                     | —        | —          | (Pawluk et al. 2014) |
| AcrIE4 | 52        | P. aeruginosa phage D3112      | Functional assays   | NP_938238.1      | I-E [Pae]                            | —                          | —                     | —        | —          | (Pawluk et al. 2014) |
| AcrIE5 | 65        | Pseudomonas otitidis mobile genetic element | Guilt-by-association [aca1] | WP_074973300.1   | I-E [Pae]                            | —                          | —                     | —        | —          | (Marino et al. 2018) |
| AcrIE6 | 79        | P. aeruginosa mobile genetic element | Guilt-by-association [aca1] | WP_087937214.1   | I-E [Pae]                            | —                          | —                     | —        | —          | (Marino et al. 2018) |
| AcrIE7 | 106       | P. aeruginosa mobile genetic element | Guilt-by-association [aca1] | WP_087937215.1   | I-E [Pae]                            | —                          | —                     | —        | —          | (Marino et al., 2018) |
| AcrIF1 | 78        | P. aeruginosa phage JBD30      | Functional assays   | YP_007392342.1   | I-F [Pae, Pec]                       | 2–3 copies interact with the hexameric Cas7f spine of the Cascade (block DNA binding) | 4-stranded antiparallel β-sheet + 2 α-helices at one side of the β-sheet | 21WS, 5SUZ, 6ANV, 6B46 | (Bondy-Denomy et al. 2013), (Guo et al. 2017), (Chowdhury et al. 2017) |
| AcrIF2 | 90        | P. aeruginosa phage D3112      | Functional assays   | NP_938237        | I-F [Pae, Pec]                       | Binds as a dimer to the Cas3 (blocks DNA binding) or spacer acquisition by the Cas1-2/3 complex (blocks adaptation) | Dimeric structure; each monomer 6 α-helices | 5GNF, 5QGH, 5B71 | (Bondy-Denomy et al. 2013), (Vorontsova et al. 2015), (Wang et al. 2016a), (Wang et al. 2016b) |
| AcrIF3 | 139       | P. aeruginosa phage JBD88a     | Functional assays   | YP_007392440.1   | I-F [Pae]                            | Binds to the Cas5f:Cas8f tail of the Cascade, mimicking DNA (blocks DNA binding) | 4-stranded antiparallel β-sheet + 2 antiparallel α-helices at either side of the β-sheet | 5GNF, 5QGH, 5B71 | (Bondy-Denomy et al. 2013), (Vorontsova et al. 2015), (Wang et al. 2016a), (Wang et al. 2016b) |
| Family | Size (aa) | Origin (characterized homolog) | Method of discovery | Accession number | CRISPR-Cas type inhibited (organism) | Mode-of-action | Structure | PDB code | References |
|--------|-----------|--------------------------------|---------------------|------------------|--------------------------------------|----------------|-----------|----------|------------|
| AcrIF4 | 100       | \( P. \text{aeruginosa} \) phage JBD26 | Functional assays | WP_016068584.1 | I-F [Pae] | Binds to the Cascade (blocks DNA binding) | — | — | (Bondy-Denomy et al. 2013) |
| AcrIF5 | 79        | \( P. \text{aeruginosa} \) phage JBD5 | Functional assays | YP_007392740.1 | I-F [Pae] | — | — | — | (Bondy-Denomy et al. 2013) |
| AcrIF6 | 100       | \( P. \text{aeruginosa} \) prophage | Guilt-by-association \([\text{aca}1]\) | WP_043884810 | I-F [Pae, Pec], I-E [Pae] | — | — | — | (Pawluk et al. 2016a) |
| AcrIF7 | 83        | \( P. \text{aeruginosa} \) prophage | Guilt-by-association \([\text{aca}1]\) | ACD38920.1 | I-F [Pae, Pec] | — | — | — | (Pawluk et al. 2016a) |
| AcrIF8 | 92        | \( \text{Pectobacterium carotovorum} \) phage ZF40 | Guilt-by-association \([\text{aca}2]\) | AFC22483.1 | I-F [Pae, Pec] | — | — | — | (Pawluk et al. 2016a) |
| AcrIF9 | 68        | \( \text{Vibrio parahaemolyticus} \) mobile genetic element | Guilt-by-association \([\text{aca}2]\) | WP_031500045.1 | I-F [Pae, Pec] | — | — | — | (Pawluk et al. 2016a) |
| AcrIF10 | 97       | \( \text{Shewanella xiamenensis} \) prophage | Guilt-by-association \([\text{aca}2]\) | KEK29119 | I-F [Pae, Pec] | Binds to the Cas7f:Cas8f tail, mimicking DNA (blocks DNA binding) | 4-stranded antiparallel \( \beta \)-sheet + 3 antiparallel \( \alpha \)-helices at one side of the \( \beta \)-sheet | 6ANW, 6B48 | (Pawluk et al. 2016a), (Guo et al. 2017) |
| AcrIF11 | 132      | \( \text{P. \text{aeruginosa}} \) mobile genetic element \( \text{P. \text{aeruginosa}} \) | Guilt-by-association \([\text{aca}1]\) | WP_038819808.1 | I-F [Pae] | — | — | — | (Marino et al. 2018) |
| AcrIF12 | 124      | \( \text{P. \text{aeruginosa}} \) | Guilt-by-association \([\text{aca}4]\) | ABR13388.1 | I-F [Pae] | — | — | — | (Marino et al. 2018) |
| AcrIF13 | 115      | \( \text{Moraxella catarrhalis} \) prophage | Self-targeting, Guilt-by-association \([\text{acrIF11}]\) | EGE18854.1 | I-F [Mbo] | — | — | — | (Marino et al. 2018) |
| AcrIF14 | 124      | \( \text{M. \text{catarrhalis} \text{phage \text{Mcat5}}} \) | Self-targeting, Guilt-by-association \([\text{acrIF11}]\) | AKI27193.1 | I-F [Mbo] | — | — | — | (Marino et al. 2018) |
| AcrIF4-F7 | 119    | \( \text{Pseudomonas citreoviolosis mobile genetic element} \) | Guilt-by-association \([\text{aca}1]\) | WP_064584002.1 | I-E [Pae], I-F [Pae] | — | — | — | (Marino et al. 2018) |
Table 2. Class 2 anti-CRISPR protein families.

| Family | Size (aa) | Origin (characterized homolog) | Method of discovery | Accession number | CRISPR–Cas type inhibited [organism] | Mode-of-action | Structure | PDB code | References |
|--------|-----------|--------------------------------|---------------------|------------------|--------------------------------------|----------------|-----------|----------|------------|
| AcrIIA1| 149       | Listeria monocytogenes prophage J0161a | Self-targeting | WP.003722518.1 | II-A [Lmo] | Recognizes nucleic acids (putative transcriptional regulation) | Dimeric structure with pseudo 2-fold symmetry; each monomer 5 α-helices + 13 βα helix at N-terminus and 3 α-helices + 23 βα helices at C-terminus (a 6 helical 2-domain) | 5Y6A | (Rauch et al. 2017), (Ka et al. 2018) |
| AcrIIA2| 123       | L. monocytogenes prophage J0161a | Self-targeting | WP.003722517.1 | II-A [Lmo, Spy] | Binds to the PAM-interacting, the WED, the HNH, and the REC2 domains (blocks DNA recognition, binding/unwinding, and cleavage, respectively) | Bent 4-stranded antiparallel β-sheet + 2 α-helices at either side of the β-sheet | 6MCB, 6MCC, 6IFO | (Rauch et al. 2017), (Jiang et al. 2019), (Liu et al. 2018) |
| AcrIIA3| 125       | L. monocytogenes prophage S1CC2482 | Self-targeting, Guilt-by-association [acrIIA1] | WP.014930691.1 | II-A [Lmo] | — | — | — | (Rauch et al. 2017) |
| AcrIIA4| 87        | L. monocytogenes prophage J0161b | Self-targeting, Guilt-by-association [acrIIA1] | WP.003723290.1 | II-A [Lmo, Spy] | Binds to the PAM-interacting, the Topo-homology, and the RuvC domains (blocks DNA recognition, binding/unwinding, and cleavage, respectively) | 3-stranded antiparallel β-sheet + 3 α-helices at one side of the β-sheet + 13 βα helix | 5XMA, 5XBL, 5VYL | (Rauch et al. 2017), (Kim et al. 2018), (Dong et al. 2017), (Yang and Patel, 2017), (Shin et al. 2017) |
| AcrIIA5| 140       | Streptococcus thermophilus (virulent) phage D4276 | Functional assays | ASD50988.1 | II-A [Sth1, Sth3, Spy] | — | — | — | (Hynes et al. 2018) |
| AcrIIA6| 183       | S. thermophilus (virulent) phage D1811 | Functional assays, Guilt-by-association [acrIAs] | MH000604 | II-A [Sth1] | — | Dimeric structure, each monomer 4-stranded antiparallel β-sheet + 8 α-helices | 6EYX | (Hynes et al. 2018) |
| 6EYY   | 103       | Human gut metagenomic libraries | Synthetic genetic circuit for screening of metagenomic libraries | LR030272 | II-A [Spy] | — | — | — | (Urbi et al. 2019) |
| AcrIIA7| 105       | Human gut metagenomic libraries | Synthetic genetic circuit for screening of metagenomic libraries | LR030270 | II-A [Spy] | — | — | — | (Urbi et al. 2019) |
| AcrIIA8| 141       | Human gut metagenomic libraries | Synthetic genetic circuit for screening of metagenomic libraries | LR030269 | II-A [Spy] | — | — | — | (Urbi et al. 2019) |
| Family | Size (aa) | Origin (characterized homolog) | Method of discovery | Accession number | CRISPR–Cas type inhibited [organism] | Mode-of-action | Structure | PDB code | References |
|--------|-----------|-------------------------------|--------------------|------------------|--------------------------------------|----------------|-----------|----------|------------|
| AcrI/A10 | 109 | Soil metagenomic libraries | Synthetic genetic circuit for screening of metagenomic libraries | LR030271 | II-A [Spy] | — | — | — | (Uribe et al., 2019) |
| AcrI/C1 | 86 | Neisseria meningitidis mobile genetic element | Guilt-by-association [aco2] | WP.049360089.1 | II-C [Nme, Cje, Geo, Hpa, Smu] | Binds to the HNH active site (allows DNA binding, blocks DNA cleavage) | 5-stranded β-bundle interspaced by 2 α-helices | 5VGB | (Pawluk et al. 2016b), (Harrington et al. 2017), (Lee et al. 2018), (Zhu et al. 2019) |
| AcrI/C2 | 123 | N. meningitidis prophage | Guilt-by-association [aco3] | WP.042743678.1 | II-C [Nme, Hpa, Smu, SauCas9, SpyCas9, FnoCas9, CjeCas9] | Binds to the bridge helix (BH)-REC1 region (blocks DNA binding) | Dimeric structure, each monomer 6-stranded antiparallel β-sheet (half-barrel structure) flanked by 2 α-helices (the C-terminal α-helix is embedded into the half-barrel) | 6J9K, 6J9L, 6J9M | (Pawluk et al. 2016b), (Lee et al. 2018), (Zhu et al. 2019) |
| AcrI/C3 | 116 | N. meningitidis prophage | Guilt-by-association [aco3] | WP.042743676.1 | II-C [Nme, Hpa, Smu] | Binds to the HNH domain opposite to the active site, and the REC lobe (binders DNA binding, blocks DNA cleavage, forces Cas9 dimerization) | 4-stranded antiparallel β-sheet + 3 α-helices at either side of the β-sheet | 6J9N | (Pawluk et al. 2016b), (Lee et al. 2018), (Zhu et al. 2019) |
| AcrI/C4 | 88 | Haemophilus parainfluenzae prophage | Guilt-by-association [aco not reported] | WP.049372635 | II-C [Nme, Hpa, Smu] | Binds to the Cas9 (blocks DNA binding) | — | — | (Lee et al. 2018) |
| AcrI/C5 | 130 | Simonsiella muelleri transfer element | Guilt-by-association [aco not reported] | WP.0264216.1 | II-C [Nme, Hpa, Smu] | Binds to the Cas9 (blocks DNA binding) | — | — | (Lee et al. 2018) |
| AcrVA1 | 170 | Moraxella bovoculi prophage | Self-targeting, Guilt-by-association [acrIF11] | WP.046701302.1 | V-A [Mbo, Asp, Lba, Fno] | — | — | — | (Marino et al. 2018), (Watters et al. 2018) |
| AcrVA2 | 322 | M. bovoculi prophage | Self-targeting, Guilt-by-association [acrIF11] | AKG19228.1 | V-A [Mbo] | — | — | — | (Marino et al. 2018) |
| AcrVA3 | 168 | M. bovoculi prophage | Self-targeting, Guilt-by-association [acrIF11] | AKG19230.1 | V-A [Mbo], I-C [Mbo] | — | — | — | (Marino et al. 2018) |
| AcrVA4 | 294 | M. bovoculi prophage | Self-targeting | WP.04699156.1 | V-A [Mbo, Lba] | — | — | — | (Watters et al. 2018) |
| AcrVA5 | 92 | M. bovoculi prophage | Self-targeting | WP.04699157.1 | V-A [Mbo, Lba] | — | — | — | (Watters et al. 2018) |
| Csx27 | 201 | Bergeyella zoohelcum | Guilt-by-association [cas13b] | WP.03685946.1 | V1-B | — | — | — | (Smargon et al. 2017) |
Table 3. Anti-CRISPR-associated (aca) genes used in the guilt-by-association approach.

| Name | Size (aa) | Accession number | References |
|------|-----------|------------------|------------|
| aca1 | 79        | XP_007392343     | (Bondy-Denomy et al. 2013) |
| aca2 | 125       | WP_019933869.1   | (Pawluk et al. 2016a) |
| aca3 | 70        | WP_049360086.1   | (Pawluk et al. 2016a) |
| aca4 | 67        | ABR13385.1       | (Marino et al. 2018) |
| aca5 | 60        | WP_039494319.1   | (Marino et al. 2018) |
| aca6 | 65        | WP_035450933.1   | (Marino et al. 2018) |
| aca7 | 68        | WP_064702654.1   | (Marino et al. 2018) |

While previous studies show the activity of CRISPR-Cas systems in vivo can clear a targeted phage in as little as 2 min (Garneau et al. 2010; Borges et al. 2018), the presence of Acr proteins seems to decrease or completely abolish bacterial immunity, classifying it as a major CRISPR-counteracting mechanism for successful phage infection and replication. However, the fast-acting nature of CRISPR-Cas limits the potential of a single phage to overcome the host's defence by Acr activity. Recently, it was shown that even though CRISPR-Cas systems are partially affected by the expression of Acr proteins, the latter are not able to confer full protection to their phage associated genome upon a single infection (Borges et al. 2018; Landsberger et al. 2018). Instead, a critical Acr concentration inside each single cell is necessary for successful host immunosuppression, allowing posterior lytic re-infection or genomic integration of temperate phages (Borges et al. 2018; Landsberger et al. 2018). It was then demonstrated that a single clonal phage population could inactivate CRISPR-Cas immunity through phage cooperation, where failed infections from ‘sacrificial Acr donors’ allow accumulation of Acr inhibitors inside a cell, which, upon a certain threshold, leads the ‘acceptor’ phages to successfully infect and amplify (Borges et al. 2018; Landsberger et al. 2018). The quantitative demand of Acr proteins for full host immunosuppression postulated that phage concentration has a key role on CRISPR evasion, which is inversely proportional to the strength of each Acr protein (Borges et al. 2018; Landsberger et al. 2018).

The existence of Acr proteins might also explain the incomplete, absent or deficient CRISPR-Cas systems found in bacteria (Stanley and Maxwell 2018). Prophage integration into the host chromosome and consistent Acr expression might result in CRISPR-Cas inactivating mutations, loss of cas genes and even complete loss of CRISPR-Cas systems (Stanley and Maxwell 2018). Interestingly, although it is evident that Acr proteins are relevant for the phage it originated from, bacteria might also benefit from the stable expression of these proteins (e.g. from prophage regions). For example, inhibition of CRISPR-Cas immunity might enhance HGT in these hosts, which can have a positive contribution to bacterial fitness upon acquisition of beneficial foreign genetic material (Bondy-Denomy et al. 2013; Jiang et al. 2013; Pawluk et al. 2014; Borges et al. 2018; Stanley and Maxwell 2018).

MECHANISMS AND STRUCTURES OF ANTI-CRISPR PROTEINS

Over the last six years, a series of studies interlacing genetic, biochemical and structural analyses have elucidated the mechanism of action of 12 Acr proteins from different families. Although many Acrs remain to be tested for anti-adaptation activity, the vast majority of the currently characterized Acr
proteins act at the interference phase by directly blocking target DNA binding or cleavage (Fig. 2). These two general modes of action are spread among both class 1 and class 2 Acr proteins, with distinct molecular mechanisms (Borges, Davidson and Bondy-Denomy 2017).

Class 1 Anti-CRISPR Proteins

The class 1 Acr proteins studied up till now all impede type I (subtype C, D, E or F) CRISPR-Cas systems (Table 1). Two mechanistic routes have been described for Acrs to perturb CRISPR interference: the most common is the direct interaction with the Cascade surveillance complex to prevent DNA binding (Bondy-Denomy et al. 2015; van Erp et al. 2015; Maxwell et al. 2016; Chowdhury et al. 2017; Guo et al. 2017; Peng et al. 2017), while the less common involves the direct interaction with the effector nuclease Cas3, which typically gets recruited upon successful target binding by the Cascade, to block DNA cleavage (Pawluk et al. 2014; Bondy-Denomy et al. 2015; Pawluk et al. 2016a; Wang et al. 2016a; Wang et al. 2016b; Pawluk et al. 2017).

A) Preventing DNA Binding via Interaction with the Cascade Complex

Steric occlusion of DNA binding

AcrF1 from P. aeruginosa phage JBD30 binds along the hexameric Cas7f spine with a stoichiometry of 2–3 molecules per Cascade complex (Bondy-Denomy et al. 2015). Several high-resolution cryo–electron microscopy and nuclear magnetic resonance (NMR) studies combined with site-directed mutagenesis indicated that AcrF1 molecules bind tightly at different positions of the P. aeruginosa Cascade complex. More specifically, two AcrF1 monomers sit on the Cas7f.4 and Cas7f.6 thumbs (Tyr6, Tyr20 and Glu31 lying on a single interaction surface of each monomer interact with the conserved Lsy85 on the Cas7f thumb), resulting in a conformational change that sterically blocks access of the crRNA guide to the target DNA, while a possible third monomer binds to a Cas7 region in close proximity to the Cas8f-Cas5f tail, which is crucial for target DNA binding (Bondy-Denomy et al. 2015; Maxwell et al. 2016; Chowdhury et al. 2017; Guo et al. 2017; Peng et al. 2017; Gabrieli et al. 2018).

Figure 1. Different discovery and testing methods of Acrs. (A) Guilt-by-association discovery method (Pawluk et al. 2016a). This discovery method is based on the strong co-occurrence and clustering of acr and aca genes through proximity and homology searches. In this example, homology searches using the acr1 gene yields its homologue acr1.1. Inspection of genes in close proximity yielded acaY.1, which in turn can be used for further iterative rounds of acr and/or aca gene discovery. Both acr and aca genes typically appear in clusters leading to the discovery of new acr and aca genes. (B) The self-targeting discovery method (Rauch et al. 2017). The presence of a self-targeting spacer (in green) within the CRISPR array hints at the presence of a (set of) acr gene(s) (in purple) somewhere within the host’s genome, often within prophage regions. (C) Low- and high-throughput functional assays to identify phage-encoded Acrs. In a low-throughput assay, individual phages are used to screen for anti-CRISPR activity in hosts with a CRISPR-Cas system targeting the phage (left) using (for example) plaque assays. High-throughput screening can be performed by transforming phage ORF libraries that are placed on a plasmid containing a protospacer. Successful transformants can be screened further to pinpoint the gene with the Acr activity within the collection of ORFs.
Figure 2. Schematic overview of the different Acrs and their mechanisms. The green boxes on the left show the different stages of CRISPR-Cas immunity. The columns indicate which CRISPR-Cas type is suppressed by which (group of) Acrs. Acrs are depicted as circles with their abbreviated names (e.g. AcrIF3 is abbreviated to IF3). A dashed line indicates a suggested role for the particular Acr or that the Acr mechanism remains to be elucidated. Note that most Acrs appear to suppress the interference stage, whereas only one Acr (AcrIF3) suppressed different stages.

DNA mimicry
While AcrIF1 exploits three different binding modes to disrupt target DNA recognition by the Cascade complex, AcrIF2 from *P. aeruginosa* phage D3112 mediates inhibition by interacting with a single site within the complex. AcrIF2 directly competes with the target DNA for a positively charged binding interface on the Cas5f:Cas8f tail between the Cas7f.6 thumb and the Cas8f hook, a region called the ‘lysine-rich vise’ (Bondy-Denomy et al. 2015). The small acidic AcrIF2 protein behaves as a DNA mimic, as the numerous acidic residues on its surface adopt a pseudo-helical distribution, resembling a double-stranded DNA (dsDNA) molecule. The interaction sites of AcrIF2 and DNA on the Cas5f:Cas8f heterodimer overlap partially, and AcrIF2 binding shoves the Cas8f hook away from the DNA-association pocket, sterically hampering the access of the dsDNA to the Cascade complex. Additional interactions of AcrIF2 with basic residues crucial for DNA binding further ensure obstruction of target DNA binding (van Erp et al. 2015; Chowdhury et al. 2017; Peng et al. 2017). Given the close proximity of the interaction sites of AcrIF2 and the third AcrIF1 monomer, when cooperating, AcrIF1 exhibits a maximum of two binding modes while AcrIF2 activity remains intact (Peng et al. 2017).

Similar to AcrIF2, AcrIF10 from *Shewanella xiamenensis* prophage also mimics DNA by occupying a region on the Cas5f:Cas8f heterodimer that closely overlaps with the binding site of AcrIF2 (possibly Cas8f K71 and R78, Cas5f R90 and Cas7f K299). However, instead of wrenching the Cas8f hook away, AcrIF10 triggers a partially closed state of the hook swinging it toward Cas7.6f (similar but smaller than the movement caused by DNA binding), displaying the conformational flexibility of this domain and implying the need of additional interactions for absolute closure of the hook. Intriguingly, AcrIF10 and dsDNA display different charge profiles on the interaction surface; nevertheless, they interact with closely overlapping regions in the Cascade complex to prevent DNA binding (Guo et al. 2017).

The first archaeal Acr protein identified, AcrID1, was shown to directly interact as a homodimer with two copies of the large subunit (Cas10d) of the type I-D Cascade complex in *Sulfolobus islandicus*. The strong negatively charged surface of this protein suggests that it may behave as a DNA mimic, such as AcrIF2. In addition, conserved residues on the surface of AcrID1, such as Glu21, Lys34, Tyr55, Glu81, Arg92 and Trp91, may have a key role in inter-protein interactions. However, the exact mechanism of AcrID1 still remains to be explored (He et al. 2018).

Unknown mechanisms
AcrIE3 and AcrIF4 from *P. aeruginosa* phage DMS3 and JBD26, respectively, have been shown to associate with the Cascade complex to hinder DNA binding, though via an unknown mechanism (Pawluk et al. 2014; Bondy-Denomy et al. 2015).

B) Preventing DNA Cleavage via Interaction with the Cas3 Nuclease

Disruption of binding to the Cascade:dsDNA chimera
Cryo-electron microscopy and X-ray crystallography demonstrated that the AcrIF3 protein from *P. aeruginosa* phage JBD5 forms a homodimer that binds to the Cas3 nuclease (Wang et al. 2016a; Wang et al. 2016b). High binding affinity was observed, since more than half of the AcrIF3 surface interacts with the Cas3 protein, forming several hydrogen bonds and hydrophobic interactions. As a consequence, the interaction sites for both
the non-complementary DNA strand and the Cascade complex are blocked. Specifically, one AcrIF3 monomer occupies the helicase domain (HD) and the Linker region of Cas3, while the other monomer relates to the C-terminal domain (CTD) (Tyr97, Trp93, and a large network of hydrogen bonds), which altogether constitute the internal cleft of the Cas3 structure. By covering this cleft, AcrIF3 disrupts association with the target DNA (non-complementary strand) and locks the ATP-dependent Cas3 nuclease in an inactive ADP-bound form (Wang et al. 2016a; Wang et al. 2016b). Noteworthy, the Cas3 effector nuclease/helicase is fused to the Cas2 protein in type I-F systems and thereby forms an integral part of the type I-F (primed) adaptation machinery, hinting at the functional link between adaptation and interference, as shown recently (Kunne et al. 2016; Staals et al. 2016; Fagerlund et al. 2017). Interestingly, AcrIF3 dimer binds to the opposite site of Cas2, thus not influencing the assembly of the Cas1-Cas2-Cas3 complex (Rollins et al. 2017). However, the dimer obstructs the recruitment of the Cascade:dsDNA chimera to Cas3, preventing the generation of precursor protospacer DNA. Consequently, AcrIF3 blocks both primed spacer acquisition and crRNA interference (Vorontsova et al. 2015; Wang et al. 2016b).

Unknown mechanisms
Akin to AcrIF3, AcrIE1 from P. aeruginosa phage JBD5 directly associates with the ATP-dependent Cas3 nuclease, without affecting the binding ability of the Cascade to the target DNA. Due to the structural homology between Cas3 proteins of type I-F and I-E CRISPR-Cas systems, it is likely that AcrIF3 and AcrIE1 either adopt distinct modes of binding to the same surface or target unrelated regions on the Cas3 protein, hindering target DNA cleavage (Pawluk et al. 2014; Pawluk et al. 2017).

Class 2 anti-CRISPR proteins
Class 2 Acr proteins have been discovered for type II (subtype A and C), type V (subtype A) and type VI (subtype B) CRISPR-Cas systems (Table 2). Almost all type II Acrs characterized to date directly interact with the Cas9 endonuclease, although by distinct mechanisms, as described below.

A) Preventing DNA Binding via Interaction with the Cas9 Protein

DNA mimicry and steric occlusion of DNA binding and cleavage
Both AcrIIA2 and AcrIIA4 from Listeria monocytogenes prophage J0161a/b have been demonstrated in vivo and in vitro to directly interact with single-guide RNA (sgRNA)-loaded SpyCas9, abolishing DNA binding and cleavage (Zhu et al. 2017; Yanga and Patel 2017). Remarkably, AcrIIA2 requires the apo form for effective inhibition. Specifically, AcrIIA2 (Asp71 and Glu72) associates with the PAM-interacting domain (Arg1335 and Arg1333), the WED domain (Gln774 and Arg778) interact with Asn19 of AcrIIA2 and the REC2 domain (Lys268 and Asp269) interact with Gln7, Thr28 and Asp30 of AcrIIA2 (Jiang et al. 2019; Liu et al. 2019). Notably, both AcrIIA4 and AcrIIA2 are not able to bind to SpyCas9 in the absence of a preloaded sgRNA, as sgRNA-binding is required for the formation of the Acr-interaction surface of SpyCas9 (Dong et al. 2017; Shin et al. 2017; Yang and Patel 2017). Similar to AcrIIA4, AcrIIA2 prevents target DNA recognition, binding and cleavage.

DNA and sgRNA mimicry
Although AcrIC2 from Neisseria meningitidis prophage was previously speculated to associate with catalytic residues of the NmeCas9 HNH domain (Pawluk et al. 2016b; Harrington et al. 2017), a recent biochemical and structural study revealed interaction with the NmeCas9 bridge helix (BH)-REC1 domain (residues 51–241) (Zhu et al. 2019). Indeed, AcrIC2 forms an homodimer, forming an acidic groove on top of the dimer. The residues in this groove (E18, N23/D24/E25 and residues 109–124) strongly bind to the arginine-rich α helix of BH (residues 56–79; mainly R62, R63, R70 and R73). As such, the AcrIC2 dimer significantly impedes sgRNA loading to apoNmeCas9, and abrogates dsDNA binding to the sgRNA-NmeCas9-AcrIC2 complex. Remarkably, AcrIC2 requires the apo form for effective inhibition, being the first Acr reported to directly interfere with the sgRNA loading to Cas9 (Zhu et al. 2019).

Dimerization of Cas9
AcrIC3 from N. meningitidis prophage hinders DNA binding and induces NmeCas9 dimerization by associating with the HNH domain and the REC lobe, respectively (Zhu et al. 2019). AcrIC3 (K532–Y540, R557–H563) interacts with a non-conserved region of the NmeCas9 HNH domain opposite to the active site (L58, N60, R33, V34 and D38 among others) (Zhu et al. 2019), allowing PAM detection but hampering complete R-loop formation (Harrington et al. 2017). Hence, the binding affinity for target DNA is decreased, while DNA cleavage is abrogated. AcrIC3 additionally associates with the REC lobe, triggering NmeCas9 dimerization with a 2:2 stoichiometry. Each AcrIC3 molecule binds to the HNH domain as well as the REC lobe of the same or another NmeCas9 molecule, forcing AcrIC3-Cas9 dimerization and preventing target DNA loading (Zhu et al. 2019).

Unknown mechanisms
Recently, AcrIC4 and AcrIC5 were discovered in a Haemophilus parainfluenzae prophage and a Simonsiella muelleri transfer element. Both were shown to impede the Cas9:sgRNA complex from binding to the target DNA, following an unknown mode-of-action (Lee et al. 2018).

B) Preventing DNA Cleavage via Interaction with the Cas9 Protein at the Catalytic Site
Like the type I AcrIF3, AcrIC1 from N. meningitidis MGE allows binding of the CRISPR interference complex to the target DNA, though hampering DNA cleavage. Biochemical and structural characterization have revealed that AcrIC1 specifically binds to the active site of the NmeCas9 HNH domain (D587, H588),
blocking cleavage of the target strand and preventing conformational changes necessary for the activation of the RuvC domain, which would theoretically catalyse cleavage of the non-target strand. Thus, the sgRNA-loaded Cas9 remains bound to the target DNA, being trapped in a catalytically inactive state. To achieve high stability of the inter-protein interaction, AcrIIIC1 additionally associates with five neighbouring residues of the HNH domain (K549, K551, D598, K603, N616). Similar to AcrIIC1, AcrIIIC2 associates with catalytic residues of the NmeCas9 HNH domain, albeit the exact mode-of-action is still elusive (Pawluk et al. 2016b; Harrington et al. 2017).

C) Preventing CRISPR-Cas Immunity via Putative Binding to RNA or DNA Molecules

Similar to AcrID1, AcrIIA1 from L. monocytogenes prophage J0161a forms a homodimer, though with an unusual two helical-domain structure. The N-terminal domain resembles the HTH motif of transcriptional factors, whilst the CTD adopts an architecture of unknown function. It is anticipated that AcrIIA1 recognizes and associates with heterogeneous RNA molecules to abolish CRISPR-Cas immunity. However, no binding to CRISPR RNA (crRNA), trans-activating RNA (tracrRNA) or their duplex has been observed. AcrIIA1 harbours a positively charged surface around the HTH region, resembling nucleic acid binding motifs of many transcriptional factors that are crucial for RNA and dsDNA recognition. Hence, it would be possible that AcrIIA1 binds to the promoter regions of crRNA or tracrRNA to hinder CRISPR-Cas immunity. However, Cas9 expression levels appeared to be unaffected by AcrIIA1 (Ka et al. 2018). The unique structure and function of AcrIIA1 reveals a novel mechanism of action yet unknown among Acr proteins, strengthening our understanding about the versatile and sophisticated ways in which these small proteins may hamper CRISPR-Cas systems (Rauch et al. 2017; Ka et al. 2018).

D) Preventing RNA Binding or Cleavage via Interaction with the Cas13b Protein

Although not associated to a phage genome, through the computational pipeline that guided the discovery of subtype VI-B CRISPR-Cas loci, the accessory protein Csx27 was recently found to repress the interference stages of its associated CRISPR-Cas system (Smargon et al. 2017). Experimental testing of Bergeyella zoohelcum Csx27 (201 aa) has demonstrated an inhibitory effect of the protein when expressed together with different Cas13b proteins, weakening their RNA interference activity. Even though important details on the mechanism of Csx27 remain to be identified, the current findings suggest a broad activity of the protein among type VI-B loci, relating to a possible regulatory mechanism of phage interference (Smargon et al. 2017).

APPLICATIONS OF ANTI-CRISPR PROTEINS

CRISPR-Cas systems have recently been scrutinised for their potential in biotechnological applications. Type II CRISPR-Cas9 systems raise special interest among the scientific community, due to their programmability and specific nuclease activity, representing the most promising tool on genome editing and modulation studied to date (Komor, Badran and Liu 2017). The use of catalytically inactive Cas9 (‘dead’, dCas9) was also previously shown to have powerful biotechnological applications. These include CRISPR interference and activation (CRISPRi and CRISPRa), modification of epigenetic marks and gene expression modulation when fused dCas9 to other metabolic key enzymes (Hilton et al. 2015). Other applications encompass dynamic genomic imaging, identification of specific genes or genomic loci, monitoring of gene copy and follow-up of chromatin formation and telomere elongation, when combining site-specific sgRNA molecules with fluorescent tagging of dCas9 (EGFt-dCas9) (Rauch et al. 2017; Liu et al. 2018). Recently, a Cas9-Assisted Targeting of Chromosome segments (CATCH) was also used for nanopore sequencing of a breast cancer gene (Gabrieli et al. 2018). Taken together, the quick emergence of CRISPR-based technologies and the continuous quest for finding new CRISPR-Cas nucleases and variants hereof indicates that more (advanced) applications are expected to be developed in the near future. However, full specificity is crucial in these applications, especially those with therapeutic purposes, as off-target events are still one of the main bottlenecks that limit the efficiency of this technology (Zhang et al. 2015).

Deeper insight in Acr inhibitory mechanisms might soon allow for precise temporal, spatial and conditional control of CRISPR-Cas systems through an ‘on-off switch’ regulation. Several AcrIIA and AcrIIC proteins were found to work as ‘off-switches’ of Cas9 activity in human cell lines (Pawluk et al. 2016b, Rauch et al. 2017). The potential of combining these natural CRISPR inhibitors with Cas9 editing systems, tuning its activity in cellular environments, could result in full optimisation of gene editing processes, substantially decreasing off-target events by allowing Acr proteins to accumulate whenever or wherever editing activity is unwanted (Pawluk et al. 2016b; Rauch et al. 2017; Shin et al. 2017; Hynes et al. 2018). Innovation in the use of Acrs to control CRISPR-Cas editing are expected to quickly emerge, as demonstrated by the combination of an AcrIIA4 hybrid with a LOV2 photosensor for the light-mediated control of genome and epigenome editing by CRISPR-Cas9 effectors in human cells (Bubeck et al. 2018). Acr-mediated inhibition was also proven to be effective towards the activity of dCas9 processes (Rauch et al. 2017; Liu et al. 2018; Nakamura et al. 2019) as well as the control of genomic circuits and gene editing with Cas9 (Nakamura et al. 2019), once again representing a promising tool for optimization of the activity of these CRISPR-based technologies and future therapeutic and biotechnological applications.

Although no research was performed yet on the possible applications of Csx27, this represents the only protein known to date to repress Cas13b, a type VI CRISPR associated protein. In contrast to most other CRISPR-Cas systems currently classified, prokaryotes carrying type VI CRISPR loci are able to target foreign RNA molecules (Smargon et al. 2017). The discovery and employment of Acr proteins able to inhibit these systems might allow modulation of future RNA-based biotechnology applications.

Acr proteins might also benefit gene drive technology. Gene drives are powerful tools to eradicate vector-borne diseases, eliminate pests (e.g. agricultural pests and invasive species) and even increase animal welfare. The technology enables the rapid dissemination of genetic mutation(s) through a population by surpassing Mendelian inheritance rules regardless of the fitness-affecting properties of the introduced mutation (Burt 2003). This is accomplished by turning heterozygous organisms into homozygotes through the incorporation of the desired mutated gene flanked by a homing endonuclease (such as CRISPR-Cas) and a corresponding guide RNA (targeting the wild-type gene) in the genome. Upon recognition of the target sequence (i.e. the wild-type gene) on the homologous
chromosome, the homing endonuclease will introduce a break at the target site. If this event is followed by homologous recombination, a cassette consisting of the mutated gene flanked by the homing nuclease and the guide RNA will replace the wild-type locus. However, one of the main technological hurdles to overcome is the current inability to effectively control the spreading of a gene drive once out in the environment (DiCarlo et al. 2015; Webber, Raghu and Edwards 2015). Acrs can be used as a molecular tool to control this spread. These proteins have been shown to reduce the efficiency of the homing nuclease in a tweakable manner (Basgall et al. 2018; Roggenkamp et al. 2018). Acrs are also envisioned to enable timed drive activation and to aid anti-gene drives in destroying the original gene drive construct (immunization) (Basgall et al. 2018). The latter can be achieved by introducing an acr encoding gene instead of a mutated gene, as part of a gene drive. This gene drive should make use of a homing endonuclease which is not suppressed by the respective acr gene. Though gene drive efficiency can be partially controlled via sgRNA design (Noble et al. 2017; Roggenkamp et al. 2018; Yan and Finnigan 2018), incorporation of Acrs in the molecular design to control spreading is advantageous over regulation via sgRNA design since Acrs work directly against the homing endonuclease whereas sgRNA based molecular principles are case specific.

Finally, Acr proteins might represent a powerful tool to enable phage therapy in CRISPR-active hosts. The emergence of multidrug resistant bacteria represents a rising scientific concern due to the possible implications of antibiotic unresponsive infections. Phage therapy poses an interesting alternative to the control of these bacterial infections (Nobrega et al. 2015), and Acr-mediated inhibition of active CRISPR-Cas systems might facilitate the employment of known phages that would be otherwise targeted by the bacterial immune system. The possibility of using studied phages, instead of the constant search and characterisation of new ones not yet targeted by the bacterial CRISPR-Cas system, might represent a therapeutic advantage and lead to faster treatment.

OUTLOOK

A limited number of Acr proteins has been identified so far, but their sequence and structural diversity is already remarkable. The discovery of new Acrs, especially those targeting CRISPR-Cas (sub)types for which Acr proteins have not yet been found, is expected to clarify the number of distinct Acr protein families and how widespread they are. The development of new Acr identification strategies will certainly be required to avoid biases created by current pipelines. Clarification of the mechanisms of multiple Acr proteins, including the characterisation of AcrV4A, AcrV4A and AcrV5A (Dong et al. 2019; Knott et al. 2019) while our study was under review, is expected to fuel the Acr-based fine-tuning of CRISPR-Cas applications, such as gene editing or gene drives.

Because bacteria and phages have co-evolved together for billions of years, it is anticipated that bacteria have developed mechanisms to counteract Acr protein activity. Possible strategies have been hinted, including the accumulation of multiple types of CRISPR-Cas systems in a single cell (e.g. type I-E and I-F systems in P. aeruginosa), mutation of cas genes (Pausch et al. 2017), or silencing of acr gene expression. Proper research on the field will certainly increase our understanding on bacterial evolution, and also expand the CRISPR toolbox for biotechnological applications.

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