Review Article

Ecology and evolution of phages encoding anti-CRISPR proteins

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https://doi.org/10.1016/j.jmb.2023.167974
Edited by Alan Davidson

Abstract

CRISPR-Cas are prokaryotic defence systems that provide protection against invasion by mobile genetic elements (MGE), including bacteriophages. MGE can overcome CRISPR-Cas defences by encoding anti-CRISPR (Acr) proteins. These proteins are produced in the early stages of the infection and inhibit the CRISPR-Cas machinery to allow phage replication. While research on Acr has mainly focused on their discovery, structure and mode of action, and their applications in biotechnology, the impact of Acr on the ecology of MGE as well as on the coevolution with their bacterial hosts only begins to be unravelled. In this review, we summarise our current understanding on the distribution of anti-CRISPR genes in MGE, the ecology of phages encoding Acr, and their coevolution with bacterial defence mechanisms. We highlight the need to use more diverse and complex experimental models to better understand the impact of anti-CRISPR in MGE-host interactions.

Introduction

In their continuous battle against their viral foes, bacteria and archaea have developed a wide range of defence mechanisms [1–4]. Amongst these defences, the CRISPR-Cas system (Clustered Regularly Interspaced Short Palindromic Repeat, CRISPR associated) provides acquired immunity against Mobile Genetic Elements (MGE), including phages [5–7]. CRISPR-Cas systems are classified in 2 classes, 6 types, 33 subtypes and 44 variants depending on the number and nature of the Cas proteins [8]. A typical CRISPR-Cas locus is composed of a set of *cas* genes and one or several CRISPR arrays. The mechanism of CRISPR-Cas immunity relies on three distinct steps: acquisition, expression, and interference. First, a fragment of MGE genetic material (protospacer) is integrated in a CRISPR array on the bacterial genome, generating a "memory sequence" which is called spacer. While there is a bias towards acquiring spacers from foreign DNA [9], the CRISPR-Cas system can sometimes acquire a spacer matching the bacterial genome or an inserted MGE. This self-targeting spacer usually results in cytotoxicity [10] upon interference. During expression, the CRISPR array is transcribed and processed as CRISPR RNAs (crRNAs), each carrying a single spacer, which associate with the Cas proteins to form a surveillance complex. Surveillance complexes can subsequently detect MGE that carry corresponding protospacers due to complementarity to the crRNA. Most CRISPR-Cas variants only bind to the protospacer if it is flanked by a Protospacer Adjacent Motif (PAM). The targeted genetic material can be RNA or single-stranded or double-stranded DNA, depending on the CRISPR-Cas system subtype [8]. In most

CRISPR-Cas subtypes, protospacer binding leads to sequence-specific cleavage. For example, CRISPR-Cas system I-F directly targets and cleaves double-stranded DNA. However, other downstream modes of action were also identified. For instance, CRISPR-Cas type V-G [11] and type VI systems [11,12] induce non-specific RNA cleavage upon target RNA recognition, while type III CRISPR systems produces secondary messengers that activate non-specific RNases [13,14], DNases [15,16] or enzymes combining DNase and RNase activity [17].

Phages can escape CRISPR immunity by acquiring mutations in the protospacer or PAM sequences [18,19]. Emergence of point mutations is particularly favoured when only a subset of the bacterial population is CRISPR immune [20]. However, such point mutations can have negative fitness consequences for the phage, depending on the gene mutated [21,22]. Moreover, at the population-level, bacteria can evolve high spacer diversity, where many different positions in the phage genome are being targeted. As a result of this diversity, phages cannot overcome CRISPR targeting mediated by several spacers through the acquisition of point mutations. Moreover, even when a phage overcomes targeting by point mutation, mismatched spacers promote the acquisition of novel spacers [23], which further increases the number and diversity of spacers inserted within host genomes. As a result, phage population is driven extinct, as shown with type I-F CRISPR-Cas system [24,25]. In response to the threat posed by CRISPR-Cas systems, phages and other MGE have evolved anti-CRISPR (*acr*) genes [26], which allow them to overcome high population-level spacer diversity [24]. Anti-CRISPR proteins (Acr) are typically small proteins that hinder one or several steps of the CRISPR-Cas machinery, such as target binding, nuclease activation or cleavage [27]. At the time of writing, 122 different Acr proteins, belonging to 92 different subtypes [28–31] have been identified. The mechanisms of actions and structure of Acr proteins have been studied quite extensively, and we direct the reader to other reviews in this special issue that cover this topic.

Acr proteins are not part of the assembled phage particle, and *acr* genes therefore need to be expressed upon the start of the infection. Hence, *acr* genes are amongst the earliest phage genes expressed upon infection, allowing them to successfully inhibit CRISPR-Cas immunity before the phage is being destroyed [32–34]. The very high expression of *acr* genes is tightly regulated by an anti-CRISPR associated (*aca*) gene, to avoid *acr* overexpression, which would disturb expression of other phage genes [32,33]. While studies on Acr proteins have mainly focused on the mechanisms of action and applications in gene engineering, we are only starting to unravel the evolutionary ecology of *acr* genes. For example, the advantages and disadvantages of carrying one or several *acr* genes or the interplay between *acr* and other phage anti-defence mechanisms are not fully understood yet. Here, we recapitulate our current knowledge of *acr* distribution among MGEs, their ecology, their evolution as well as their coevolution with host defence systems.

Abundance and distribution of acr genes

CRISPR-Cas systems are found in 36 % of bacteria and 75 % of archaea [35], and their prevalence is particularly high in environments with high viral abundance and low viral diversity [36]. Given that bacterial CRISPR arrays often encode spacers matching local phage populations [37–39], one may expect *acr* prevalence to mirror that of CRISPR-Cas systems in their hosts. However, the prevalence of currently verified *acr* genes is much lower than CRISPR-Cas system abundance. Indeed, to date, *acr* genes homologs have been found in only 3.4 % of virulent viruses' genomes, 2.8 % of archaeal genomes and 7.4 % of bacterial genomes [40]. Moreover, most identified Acr proteins display activity against the CRISPR-Cas types I-F and II-A, while for most other CRISPR-Cas types, no Acr proteins have yet been identified. This bias in the identification of Acr subtypes may be reflective of the stronger research focus on CRISPR-Cas subtypes that have been well characterised and for which biotechnology applications have been developed. The apparent paucity of *acr* genes might be explained by the difficulties to identify them through computational approaches. Indeed, while CRISPR-Cas systems can be identified by mining genomes for the presence of CRISPR arrays [41] or Cas homologs [42], *acr* genes display extensive sequence variability, which greatly complicates their identification [27,43]. New methods were developed to identify new *acr*, such as guilt-by-association with the more conserved *aca* gene [44,45], identification of self-targeting spacers [46] or functional activity high-throughput screening of metagenomic databases [47]. More recent large-scale analyses uncovered a wider range of putative *acr* candidates [40,48,49].

Huang and collaborators built a database of putative *acr* genes called AcrDB [40]. They used a combination of three bioinformatics tools, based on gene neighbourhood, amino acid composition and evolutionary conservation, to scan genomes of bacteria, archaea, and prokaryotic viruses, thus identifying almost 40,000 operons containing *acr* or *aca* candidates. Candidates were found in 26 % of archaeal genomes, 18 % of bacterial genomes and 58 % of viral genomes, but none have been validated experimentally yet. This approach might be too restrictive as it relies heavily on the genomic context of the analysed genes. To remove this constraint, machine-learning [48] or deep-learning [49]

approaches were developed based solely on properties of protein sequences, such as length, hydrophobicity or isoelectric point. Even without relying on hallmarks of Acr (*e.g.*, presence of *aca* genes or self-targeting spacers), these methods were able to retrieve the complete dataset of AcrDB as putative Acr proteins [49], and to further expand the number of candidates [48,49]. For instance, Gussow *et al*, analysed the sequences of more than 180 million proteins of prokaryotes and prokaryotic viruses, which uncovered 2,500 families of almost 17,000 predicted Acr proteins [48]. Interestingly, only 10 % of these families were encoded on sequences from viral origin (i.e. non-integrated into prokaryotic genomes). This finding is consistent with the currently validated *acr* which were mainly found in MGE integrated in bacterial genomes [28–31]. However, only a minor part of candidates identified through large-scale analyses were experimentally verified to act as Acr proteins [48,49], and these findings must therefore be considered with caution.

Acr specialism versus generalism

Most identified Acr proteins are effective against a single CRISPR-Cas subtype and are often highly specific to a CRISPR-Cas system from a single species [44,49–55]. A recent bioinformatic analysis suggested that *acr* genes can cluster in so-called "anti-defence islands" in phages and prophages genomes [55]. Moreover, in the Gussow *et al* database, almost 30 % of the viral strains carrying putative *acr* carried two or more *acr* genes [48], consistent with previous observations made in phages and prophages from *Pseudomonas, Neisseria, Listeria, Moraxella, Streptococcus, Pectobacterium* and *Sulfolobus* [26,45,46,50,52–59]. However, the consequences of carrying multiple *acr* genes on phage infectivity and fitness are still unclear and may vary, depending on the combination of *acr* genes.

When phages carry multiple *acr* genes, these Acr often target the same CRISPR-Cas subtype, suggesting that phages evolved host specialisation [26,45,46,50,52–59]. Generally, phages carry between 2 and 4 different *acr* genes, although notable exceptions exist, such as the archaeal virus SIRV2 which possesses 12 *acrID1* paralogues [57]. Having several *acr* genes that block the same CRISPR-Cas subtype through different molecular mechanisms (*e.g.*, AcrIF1 preventing surveillance complex from binding to target DNA and AcrIF3 preventing Cas3 from being recruited to the DNA-bound surveillance complex [60]) could potentially provide synergistic protection against CRISPR immunity, although there is no experimental evidence of this. Conversely, different Acr with similar molecular targets (*e.g.*, AcrIIA2 and AcrIIA4, both preventing Cas9 from binding to target DNA [61]) may biochemically compete with each other. Finally, phages might benefit from encoding two Acr proteins that provide advantages in different contexts. Indeed, temperate phages can produce an Acr protein that is active during the lytic cycle and produce a different Acr protein during lysogeny that induces long-term downregulation of the CRISPR-Cas system. For instance, AcrIIA1, found in *Listeria monocytogenes* temperate phages, promotes Cas protein degradation which efficiently protects the prophage during lysogeny but cannot protect the phage during lysis [54]. However, *acrIIA1* is always found together with *acr* genes that are efficient during lysis, such as AcrIIA2 that blocks Cas9 DNA binding [54]. Similarly, a prophage identified in Moraxella *bovoculi strain* 58,069 encodes AcrVA2, which downregulates Cas12a mRNA, as well as AcrVA1 that cleaves Cas12a crRNA [62].

Some phages have evolved more generalist strategies by carrying several *acr* genes with distinct specificities, providing immunity against different CRISPR-Cas subtypes [45,50,55,58,59]. These phages likely have a broader host range, as they are able to infect a panel of hosts carrying a diversity of CRISPR-Cas systems. Moreover, some bacterial strains encode more than one CRISPR-Cas subtype [63] and carrying several *acr* genes with distinct specificities allows phages to access these hosts. In addition, some Acr proteins can block the same CRISPR-Cas subtype across distinct species [44–46,51–55], such as AcrIF15 that inhibits type I-F CRISPR-Cas systems from *Pseudomonas aeruginosa*, *Pectobacterium atrosepticum* and *Serratia* sp. [55]. Moreover, in some instances Acr proteins have a broad range of inhibition, as they can block different (sub)types of CRISPR-Cas systems [44,45,51,54,55,59], such as AcrIF6 that inhibits both type I-F and I-E CRISPR-Cas systems [44], or AcrVA3 that blocks both type V-A and type I-C CRISPR-Cas systems [45]. However, such Acr generalism may be limited. These limitations might result from structural differences between Cas proteins of distinct subtypes or from different bacterial species, which may hinder Acr binding, but overall, what determines these limitations is not well understood. For instance, AcrIIA1 can block the type II-A CRISPR-Cas systems from *Streptococcus pyogenes* and *Staphylococcus aureus* and the type II-C systems from four other species but is unable to block the type II-C system from *Corynebacterium diphtheriae* [54]. In addition, a recent study showed that phage DMS3m carrying *acrIF2/C2* (which has dual inhibition specificity against type I-F and type I-C systems) is less efficient when infecting *P. aeruginosa* carrying both type I-C and I-F, compared to a strain that carries two type I-C systems or a single I-F system [59]. Interestingly, a novel generalist mode of action has been recently described for AcrIII-1 [64]. This Acr protein degrades cyclic tetra-adenylate (cA4), the secondary messenger produced by the Cas10 protein (type III CRISPR-Cas system) that activates

some specific defence enzymes. Since this Acr protein does not directly interact with one of the CRISPR-Cas components, it can block any CRISPR-Cas system that use cA4 as a messenger. Interestingly, since cA4 is also used as a secondary messenger in CBASS (Cyclic nucleotide-Based Antiphage Signalling System) defence system, cross-talks between CBASS, CRISPR-Cas and their respective inhibitors may happen [65,66], but this hypothesis has not yet been experimentally confirmed. Overall, our understanding of the costs and benefits of carrying several similar or different *acr* genes is limited, and further studies are needed to better characterise them. A general prediction is that the composition of the host population is likely to determine whether *acr*-associated benefits can outweigh their costs. In the context of a host population that is composed of diverse CRISPR-Cas subtypes, intuitively phages with broad-range Acr would reproduce most rapidly because they can infect a greater diversity of hosts. However, they may also carry the greatest costs. Optimal foraging theory suggests that the optimal strategy often consists of a restricted infectivity range to reduce associated costs [67,68]. Hence, despite the presence of a bacterial population with multiple CRISPR-Cas systems, phages with low numbers of *acr* genes may be favoured, if carrying *acr* genes is costly. Therefore, to understand how host community composition drives the evolution of the *acr* loci in phage genomes, it is critical to understand the costs and benefits of *acr* genes for the phage.

Costs and benefits of acr genes and their impact on phage epidemiology

Sustaining the high expression of *acr* gene at the onset of infection requires a lot of cell resources [32–34], and therefore, one could expect that carrying *acr* genes confers a fitness cost to the phage. Surprisingly, when infecting bacteria lacking a CRISPR-Cas system, phage DMS3mvir without *acr* genes has the same fitness as isogenic variants carrying *acrIE3*, *acrIF1* or *acrIF4*, suggesting that the production of Acr proteins does not come with high costs [69]. In fact, the tight regulation of *acr* expression by *aca* genes [32,33], or through autorepression [70], likely alleviates potential fitness costs induced by the expression of *acr* genes. In the absence of repression, *acr* overexpression prevents the correct transcription of downstream phage genes, thus impairing phage replication. Indeed, deletion of the *aca1* gene in *P. aeruginosa* temperate phage JBD30 [33], or the autorepressing N-terminal domain of *acrIIA1* in *L. monocytogenes* temperate phage ΦA0006, ΦA118 and ΦJ1061A [70], results in important fitness costs for the phage, even in a host without a CRISPR-Cas system, driving the evolution of phage variants with attenuated *acr* expression due to promoter mutations.

Encoding Acr proteins gives an evident advantage to the phage when infecting a host with a CRISPR-Cas system, but the full extent of this fitness benefit depends on the pre-existence of spacers targeting the phage. Phage can readily acquire mutations in their protospacer or PAM sequence to escape CRISPR-targeting by their host [18], thus leaving bacteria with mismatched spacers. These mismatched spacers can still benefit bacteria by powering novel spacer acquisition, a phenomenon called “primed acquisition” [23]. However, some Acr proteins can prevent primed spacer acquisition, as shown in an artificial plasmid-based system in *E. coli,* where AcrIF1 to 5 inhibited spacer acquisition by *P. aeruginosa* type I-F system [71] and in *P. aeruginosa* during challenge with a lytic phage, where spacer acquisition in the type I-F system was reduced 100- to 1000-fold when phages encoded Acr proteins [69]. When temperate phages enter lysogeny, priming can induce the acquisition of detrimental self-targeting spacers that match the integrated prophage [72]. If the prophage carries an *acr* gene, the acquisition of self-targeting spacers is alleviated, which strongly benefits both the phage and the host. Therefore, even when the phage is not perfectly targeted by the host CRISPR-Cas system, *acr* genes favour phages transmission both horizontally and vertically by preventing primed spacer acquisition [72].

When the host already carries a spacer with a perfect match, phage infection success rate is very low in the absence of *acr* genes [69,73]. Therefore, it seems very unlikely that a targeted phage can establish lysogeny in the absence of an *acr* gene, which is consistent with the frequent identification of *acr* genes in prophages targeted by their host CRISPR-Cas [45,46,52,55,58,59,74]. To inhibit the host immune mechanism, Acr proteins need to be produced sufficiently rapidly to inactivate the host CRISPR-Cas system before it cleaves phage genetic material [73,75]. However, in the case of AcrIF1, AcrIF4 and AcrIIA4, Acr production rarely outpaces CRISPR-Cas activity and infection often fails initially. Nonetheless, even if the infection fails, the rapid expression of the *acr* gene concomitant to the entry of the phage genome allows the production of some Acr proteins that can leave the cell in a transient immunosuppressed state after the phage genome has been eliminated. A subsequently infecting phage can benefit from this immunosuppression and successfully amplify on the host bacterium (Figure 1A). Since the immunosuppressed bacterium will eventually revert back to its CRISPR-immune state, the second infection needs to happen quickly enough, which can only occur at high phage densities. As a result, the phage population can only grow if its initial density is above a certain threshold [73,75]. This cooperation between successive phage infections relies on fast and strong expression of *acr* genes, and therefore, environments that disfavour *acr* expres-

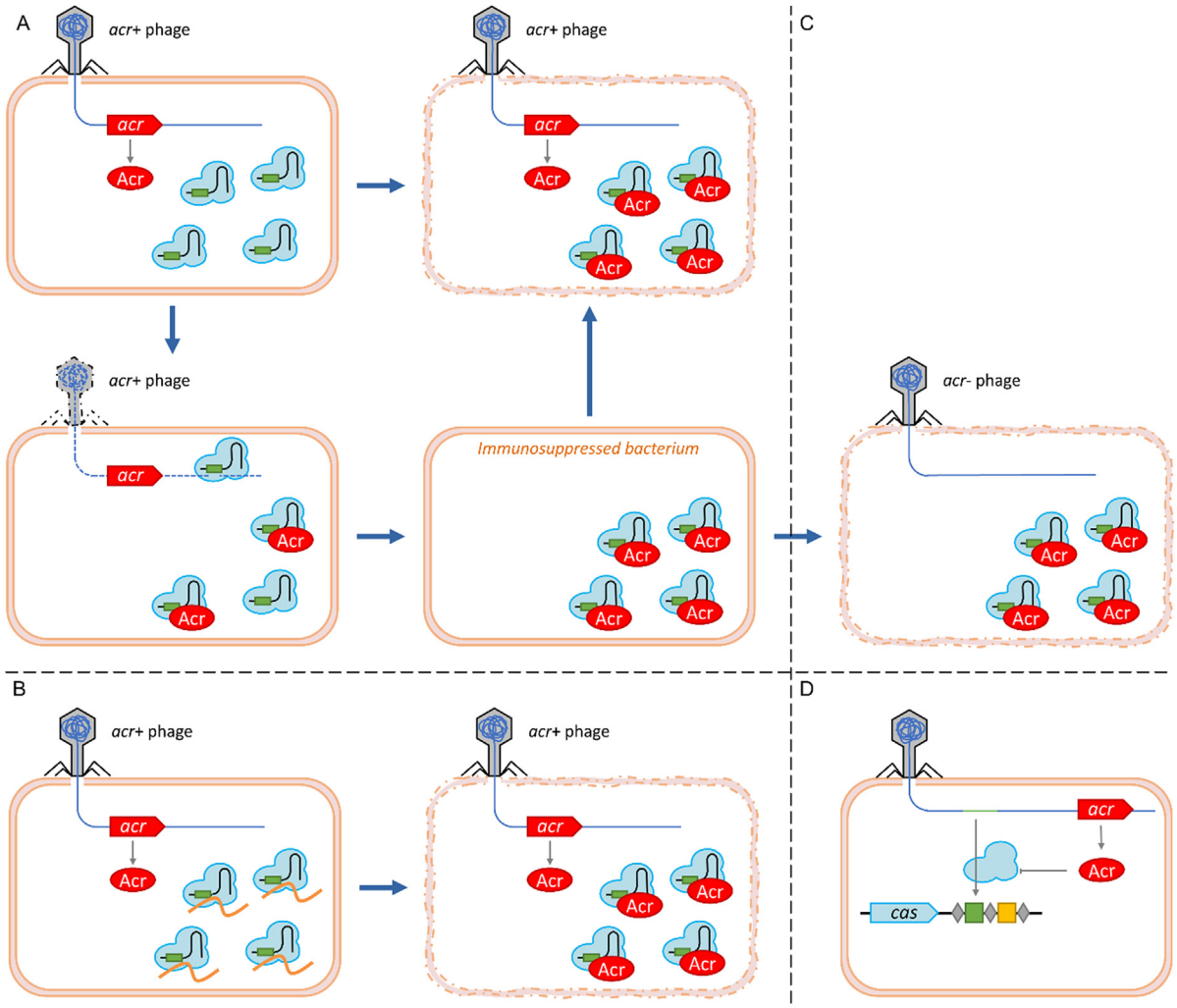

Figure 1. Phages benefit from acr genes through multiple mechanisms. A. Acr-carrying phages cooperate to lyse bacteria through the induction of immunosuppression. Acr proteins need to be produced in a timely manner to overcome CRISPR-Cas immunity. Even upon failure, the Acr proteins produced during a first failed infection inactivate some CRISPR-Cas surveillance complexes and turn the cell into a transitory immunosuppressed state. A second phage can take advantage of this immunosuppressed state to successfully replicate. B. Acr blocking RNA-targeting CRISPR-Cas system do not require cooperation to lyse bacteria (orange: RNA). With this type of CRISPR-Cas system, the phage genetic material is not cleaved and can stay in the cell until the Acr proteins are produced in sufficient quantities to inactivate the CRISPR-Cas system. This mechanism only requires a single infection to succeed. C. Acr-induced immunosuppression can be exploited by other phages that benefit from Acr proteins without encoding them. D. Acr can inhibit spacer acquisition, thus protecting non-targeted phages from acquired CRISPR-Cas immunity.

sion are expected to impair phage infectivity. For example, in *P. aeruginosa*, sub-inhibitory doses of antibiotics inhibiting protein translation can prevent the immunosuppression mediated by AcrIF1 and therefore abolish phage cooperation and replication [76]. In contrast, a recent study on AcrVIA1, which inhibits an RNA-targeting CRISPR-Cas system, showed that phage ΦLS46 can amplify on *Listeria seeligeri* independently of the initial phage load [77]. While DNA-targeting CRISPR-Cas systems eliminate invading phage DNA, the *L. seeligeri* type VI-A CRISPR-Cas system indiscriminately cleaves phage and bacterial RNA but leaves the phage genetic material intact. A slow but continuous expression of *acrVIA1* might then be sufficient to reach the Acr threshold necessary to fully overcome the CRISPR-Cas system, thus removing the need for phages cooperation (Figure 1B). Phage population dynamics thus appears to depend on the Acr mode of action.

Community-wide benefits of acr genes

Considering the important benefits provided by *acr* genes with no measurable fitness costs detected so far [69], one may wonder why most phages do not carry any *acr* genes or why some phages encode weak Acr inhibitors (*i.e.*, Acr protein that provides low success rates for the initial infection) instead of strong ones (that provide a higher initial infection success rate). An investigation conducted with *P. aeruginosa* and its virulent phage DMS3vir, either without *acr,* with a weak *acrIF4* or with a strong *acrIF1* [69], showed that phages without *acr* genes are protected from extinction in the presence of Acr-phages through a dual mechanism. First, Acr-phages produce immunosuppressed hosts, which can be directly exploited by phages without *acr* genes for successful replication (Figure 1C). Second, Acr-phages limit the evolution of CRISPR-immunity by preventing overall spacer acquisition which indirectly protects any invading phages. Interestingly, exploitation is greater with phages carrying the strong *acrIF1* compared to the weak *acrIF4*, presumably because AcrIF1 induces a stronger and more durable immunosuppression state [69]. These data suggest that exploitation of Acr proteins as a "public good" allows for the survival of phages lacking *acr* genes when facing a CRISPR-immune host. Based on these findings, we can hypothesise that weak *acr* may have been positively selected, despite their lower efficiency, because they are less prone to exploitation than their strong counterparts. However, these results were obtained with isogenic phages, and further studies are needed to evaluate to which extent the benefits of *acr* genes can be shared between different phages or MGE. For example, the presence of *acr* genes in prophages can make the host sensitive to other phages that are normally targeted by the CRISPR-Cas system [26]. This example of exploitation provides a clear disadvantage for the Acr-encoding phage as it allows other phages to lyse its host, which raises interesting questions on how selection acts on *acr* genes in this context.

Coevolution between acr and bacterial defence systems

The presence of an *acr* gene in the infecting phage genome greatly reduces the fitness benefits that CRISPR-Cas provides to the bacterial host [69]. This strong selection pressure is therefore expected to lead to bacterial evolution to overcome Acr inhibition, and potentially subsequent cycles of co-evolution. Although only few studies have addressed (co–)evolutionary interactions between Acr and CRISPR-Cas, here we will discuss potential (co–)evolutionary scenarios that emerge from the evidence provided by these studies.

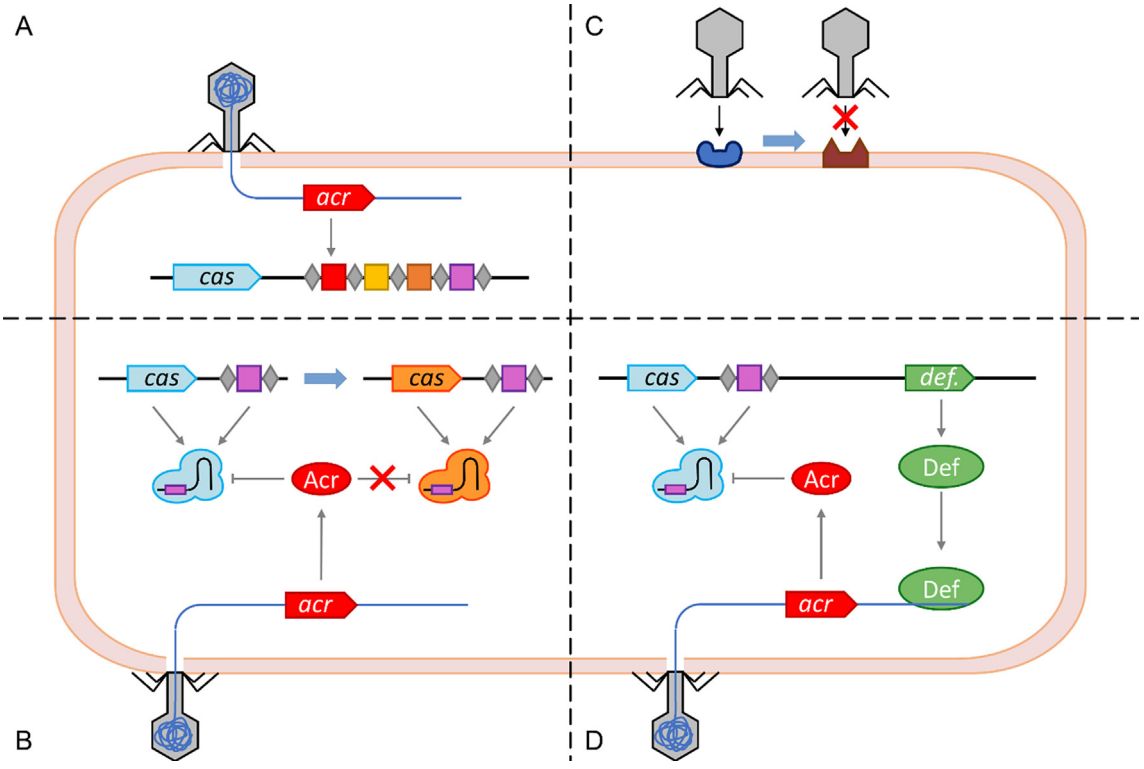

Figure 2. Strategies to escape Acr phages. A. Acquisition of spacers specifically targeting the acr gene. B. Mutations in Cas proteins to evade inhibition by Acr. C. Evolution of mutations in surface receptors, which prevents phages adsorption. D. Encoding of several anti-phage defence mechanisms (Def) in addition to CRISPR-Cas system.

An escape strategy for bacteria may consist in acquiring spacers that target the *acr* gene itself (Figure 2A). The artificial implementation of this strategy in *Streptococcus thermophilus* effectively protected bacteria against the virulent phages D1126 carrying *acrIIA5* and D3288 encoding AcrIIA6 [78]. In response, phages evolved truncated *acr* genes that lost the protospacer. Although these truncated Acr proteins lost their ability to inhibit DNA cleavage, they retained their capacity to prevent spacer acquisition. While there is no direct evidence that CRISPR-immunity can naturally evolve through the acquisition of *acr*-targeting spacers, truncated homologs of *acr* genes were identified in phages and prophages genomes, suggesting that this phenomenon may occur in nature [78]. An alternative strategy for bacteria to escape Acr-phages may consist in the modification of Cas proteins to prevent Acr binding and inhibition (Figure 2B). While there is no direct evidence of this evolutionary strategy, Acr efficiency can be disturbed by variations in Cas proteins. For instance, AcrIF20.1 efficiently blocks the *P. atrosepticum* type I-F CRISPR-Cas system but very weakly inhibits *Serratia* sp. type I-F CRISPR-Cas system, despite 80 % sequence identity between the interference proteins of the two systems [55]. Modifications of Cas proteins may in turn drive the evolution of Acr proteins that restore effective inhibition, or the acquisition of additional *acr* genes in phage genomes that are active against modified Cas proteins. However, it remains to be seen if such coevolutionary scenario can be observed in experimental systems. In the case of Acr proteins that do not directly interact with Cas proteins, such as AcrIII-1 which degrades cA4 secondary messenger produced by Type-III CRISPR-Cas systems [64], mutation of Cas proteins may be more constrained, as they would need to result in the production of modified secondary messenger that escapes degradation by Acr but retains the ability to effectively activate downstream effectors.

Finally, bacteria could evade Acr-phages by evolving CRISPR-independent phage resistance, by acquiring mutations modifying the phage receptor (Figure 2C) [69]. While surface modification is an efficient resistance mechanism in laboratory settings [1,2,4], it can confer substantial fitness costs outside the test tube and therefore may not be positively selected in natural environments [79]. Bacteria may also deploy alternative defence mechanisms to restrict Acr-phages (Figure 2D). Anti-phage defence discovery has been exponential over the last few years [80], and most bacterial genomes harbour several defence mechanisms [81]. For instance, the high prevalence of restriction-modification and CRISPR-Cas systems in bacterial genomes (respectively, 80 % and 40 %) suggests that an important proportion of bacteria carry both defence systems [81]. Similarly, phages can encode multiple anti-defence mechanisms. In addition, anti-defence genes could protect phages against multiple defence systems. For instance, AcrIII acting on cA4 levels was suggested to inhibit both cA4-relying type III CRISPR-Cas systems and CBASS mechanisms happen [65,66]. As a result, coevolution between bacteria and phages is likely influenced not only by the CRISPR-Cas-Acr interplay, but also by the wider interactions occurring between the bacterial immune network and viral anti-defence repertoires.

Conclusions and outlook

Since their initial discovery 2013, the Acr field has been very productive, with a strong focus on identification of new candidates, biochemical characterisation and repurposing as genetic engineering tools. However, the ecology and evolution of *acr* genes has received less attention, with only a handful of studies specifically focusing on that aspect. While our understanding of CRISPR-Cas and Acr mechanisms suggests that CRISPR-Acr interactions are important drivers of phage-bacteria coevolution, many questions remain on the evolution of *acr* themselves.

First, most observations on the ecological and evolutionary aspects of *acr* were conducted on type I-F CRISPR-Cas and AcrIF. Acr with different biochemical mode of actions may lead to different phage population dynamics. For instance, phages that encode AcrVIA1 do not require cooperative infections to generate an epidemic [77], unlike phages encoding AcrIF1, AcrIF4 or AcrIIA4 [73,75]. Moreover, enzymatically active Acr (*i.e.*, one Acr protein can consecutively act on several targets), like AcrVA1 [45], AcrVA5 [52] or AcrIII-1 [64], may lead to different population dynamics compared to stoichiometric Acr inhibitors (*i.e.*, one Acr protein only interacts with a single molecular target).

Second, most studies have been carried out using simple models, with one phage carrying a single *acr* infecting one host with a single CRISPR-Cas system. To understand the costs and benefits associated with *acr* generalism (either through several different *acr* or through a generalist *acr*) versus specialism, more complex experimental models are needed, with multiple *acr* genes and CRISPR-Cas systems.

Third, Acr interactions with other anti-defence mechanisms have never been studied to the best of our knowledge. In bacterial genomes, defence systems can cluster together [82,83], and these different mechanisms form layered lines of defence against phage invasion [84]. In a comparable way, it has been suggested that *acr* genes and other anti-defence genes, such as anti-restriction modification, may be clustered in anti-defence islands [55]. Potential synergies or antagonisms between

acr genes and these other anti-defence mechanisms have not yet been investigated. In addition, interactions between *acr* genes and genes not classified as anti-defence system remain to be investigated. For instance, some phages encode hijacked homologs of AmrZ, a bacterial alginate regulator that reduces CRISPR-Cas expression [85]. Engineered phages carrying this gene can efficiently block CRISPR-Cas expression during lysogeny but not during lysis. This is reminiscent of AcrVA2 that downregulates *cas* gene expression [62] and AcrIIA1 that promotes Cas protein degradation [54]. Indeed, both AcrVA2 AcrIIA1 protect the phage from CRISPR-Cas system during lysogeny, but as they are inefficient during lysis, the phage needs a second *acr* gene (or an alternative strategy) to establish infection [54,62]. Therefore, evaluating possible complementarity between *acr* genes and other genes impacting CRISPR-Cas systems could shed light on new phage infection strategies.

Fourth, the impact of *acr* genes on bacterial populations has mainly been studied in the death-survival scope. Since CRISPR-Cas systems are negatively associated with indicators of horizontal gene transfer (HGT) [86,87], the presence of Acr proteins might have broader consequences on host evolution. For example, the presence of *acr* genes, either in *cis* or in *trans*, facilitates plasmid transfer between hosts [53]. By favouring gene flow, HGT represents one of the major drivers of bacterial evolutionary innovation. In particular, HGT mediates the acquisition of virulence factors [88] or antibiotic resistance genes [89]. However, the impact of *acr* genes on these phenotypes is yet to be unravelled. For instance, in *P. aeruginosa,* the number of antibiotic resistance genes was shown to be uncorrelated with *acr* gene presence in one study [87], but the presence of *acr* genes was strongly associated with some types of antibiotic resistance genes in another study [90]. Considering the impact that virulence and antibiotic resistance can have on global health and disease, studying the long-term impact of *acr* genes on bacteria is of prime importance.

CRediT authorship contribution statement

Benoît J. Pons: Conceptualization, Writing – original draft, Writing – review & editing. **Stineke van Houte:** Conceptualization, Writing – review & editing, Funding acquisition, Supervision. **Edze R. Westra:** Conceptualization, Writing – review & editing, Funding acquisition, Supervision. **Anne Chevallereau:** Conceptualization, Writing – review & editing.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

B.J.P. was supported by funding from the UK Biotechnology and Biological Sciences Research Council (BB/S017674/1) awarded to S.v.H. S.v.H. also acknowledges funding from BBSRC grant BB/R010781/1 and EPSRC grant EP/X026507/1, and E.R.W received grants from the European Research Council under the European Union's Horizon 2020 research and innovation programme (ERC-STG-2016-714478). A.C. received funding from IdEx Université Paris Cité (ANR 18 IDEX 0001).

Received 27 October 2022;
Accepted 14 January 2023;
Available online 20 January 2023

Keywords:
Bacteriophage;
Bacteria;
Phage-bacteria interactions;
CRISPR-Cas;
Anti-CRISPR proteins

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