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David Bikard, Rodolphe Barrangou

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David Bikard[1] & Rodolphe Barrangou[2]

[1] Synthetic Biology Group, Microbiology Department, Institut Pasteur, Paris 75015, France, david.bikard@pasteur.fr

[2] Department of Food, Processing and Nutritional Sciences, North Carolina State University, NC, rbarran@ncsu.edu

Corresponding authors: David Bikard & Rodolphe Barrangou

Highlights

- Self-targeting by CRISPR-Cas systems is typically lethal in bacteria
- CRISPR-Cas systems can be repurposed to drive programmed bacterial death
- CRISPR arrays and Cas nucleases can be delivered to target organisms using phages
- There is a need to engineer delivery vectors to enable CRISPR-based antimicrobials

Abstract

Although CRISPR-Cas systems naturally evolved to provide adaptive immunity in bacteria and archaea, Cas nucleases can be co-opted to target chromosomal sequences rather than invasive genetic elements. While genome editing is the primary outcome of self-targeting using CRISPR-based technologies in eukaryotes, self-targeting by CRISPR is typically lethal in bacteria. Here, we discuss how DNA damage introduced by Cas nucleases in bacteria can efficiently and specifically lead to plasmid curing or drive cell death. Specifically, we discuss how various CRISPR-Cas systems can be engineered and delivered using phages or phagemids as vectors. These principles establish CRISPR-Cas systems as potent and programmable antimicrobials, and open new avenues for the development of CRISPR-based tools for selective removal of bacterial pathogens and precise microbiome composition alteration.

Clustered regularly interspaced short palindromic repeats (CRISPR) together with CRISPR-associated (Cas) proteins constitute the adaptive immune system of prokaryotes [1]. Over the past 10 years, they have captured the attention of the scientific community in many ways, spanning their role in driving the co-evolutionary interplay between bacteria and their viruses, and the development of CRISPR-based technologies [2–4]. Most of the applications rely on the discovery of programmable RNA-guided nucleases among the CRISPR associated (Cas) proteins. By designing the sequence of a small CRISPR guide RNA, scientists are readily able to direct these nucleases in an extremely precise manner to virtually any genomic locus of interest to drive a variety of molecular outcomes.

The diversity of CRISPR-Cas systems has been reviewed elsewhere [5]. Here, we will mostly focus on type I and II CRISPR-Cas systems, which have been used to selectively kill bacteria. In nature, Type I systems are the most widespread and hinge on a multi-protein effector complex, the CRISPR-associated complex for antiviral defense (CASCADE)[6], which uses the Cas3 exonuclease for processing of invasive DNA [7]. This is in contrast to Type II systems which rely on the potent signature effector endonuclease Cas9 to generate double stranded DNA breaks [8–10]. Conveniently, the machinery of CRISPR-Cas systems can be ported as a two component system comprising the protein Cas9 and a single guide RNA (sgRNA) mimicking the dual native crRNA:tracrRNA complex typically found in nature [10]. DNA breaks generated by Cas9 can be repaired, leading to precise alteration of the DNA sequence at the exact site of cleavage, essentially *editing* DNA with accuracy. In addition to sequence alteration, other Cas9-based technologies have recently been developed to precisely control gene expression, modify the epigenetic state of a sequence, fluorescently tag genomic loci, and carry out high-throughput genetic screens [2,11,12]. Even though most of the on-going efforts focus on eukaryotic applications of CRISPR-based technologies, CRISPR-Cas systems afford tremendous opportunities in bacteria, where either endogenous or heterologous CRISPR-Cas systems can be readily repurposed for a variety of applications, including genome editing and control of gene expression [13–16]. We focus here on the use of both type I & II CRISPR-Cas systems as programmable antimicrobials. We discuss how they can be readily directed to target undesirable sequences such as antibiotic resistance and virulence genes, with the purpose of eradicating pathogenic bacteria, or as a means to destroy the undesirable plasmids they occasionally carry [17].

Self-targeting by CRISPR-Cas systems in nature

While CRISPR-Cas systems are functionally designed to target invasive nucleic acids, they can occasionally sample chromosomal DNA from their native host. Indeed, while most CRISPR spacers match phage and plasmid DNA, some can show homology to chromosomal sequences [18]. An early report from Sorek and colleagues actually noted that in nature some CRISPR-Cas systems carry

spacers that perfectly match sequences encoded on the host chromosome [19]. While some of these self-targeting spacers match prophages and other mobile elements such as transposons, others are found to match genes surprisingly belonging to the core genome. Nonetheless, in almost all of these cases, the authors were able to identify clues that the CRISPR-Cas system has been somehow inactivated by mutations in the *cas* genes or CRISPR array, or mutations altering the targeted sequences. Mutations in the target sequence that block CRISPR immunity typically occur in the protospacer-adjacent motif (PAM) (Horvath et al., 2008), or within the seed sequence [20,21], which encompasses the cleavage site and drives the formation of the crRNA:targetDNA R-loop for cleavage [22]. A model was thus proposed where the CRISPR-Cas systems sometimes capture self-targeting spacers "by mistake" and can only survive such events if the system is functionally inactivated. A study investigating the spacer acquisition process in the *Streptococcus thermophilus* model system showed that acquisition from chromosomal sequences can occur, but established that this auto-immune sampling is rare, and that when it does occur, these genotypes do not remain in the bacterial population [23]. Specifically, the authors were able to detect acquisition from chromosomal sequences at a frequency of 0.04% (120 of 443,871 acquired spacers), and showed that these self-targeting events could only be detected at one time point, and disappeared within a day from the population, presumably because acquisition of self-targeting spacers is lethal. Several mechanisms enable CRISPR-Cas systems to preferentially acquire spacers from foreign DNA, including primed adaptation [24] and acquisition from DNA fragments generated after the processing by exonucleases of double strand breaks, or phage DNA termini [25,26]. Despite these mechanisms, it is possible to detect lethal acquisition events in natural CRISPR-Cas systems [27].

The primary outcome of self-targeting is cell death

The first report of cell death induced by a CRISPR-Cas system targeting a sequence on the bacterial chromosome was published by Qimron and colleagues. In this study, they investigated the consequences of directing the native type I-E CRISPR-Cas system from *Escherichia coli* to an integrated lambda prophage [28]. They observed that inducing the CRISPR-Cas system led to the death of 98% of the cells in the population, and already speculated at the time that cell death was the result of chromosomal DNA degradation by the Cas enzymatic machinery. It was later shown that self-targeting by Type I systems efficiently killed bacteria regardless of the target location [29], and can lead to the excision of large pieces of DNA in the target region [30]. In Type I systems, the CASCADE complex binds the target and recruits the Cas3 exonuclease leading to extensive DNA degradation [31,32]. This is mechanistically different from Type II systems where Cas9 cleaves DNA endo-nucleolytically, by cutting target DNA exactly 3 nt away from the 3' edge of the targeted proto-spacer [8], using two nickase domains [9,10]. Both CRISPR-Cas types were nonetheless shown to have

the ability to cure plasmids, or kill bacteria when reprogrammed to target the chromosome (Figure 1a) [33].

The ability to kill a population of bacteria based on its sequence using the type II system from *S. pyogenes* was used as a means to select for the introduction of mutations [14], providing the first evidence of CRISPR-mediated genome editing in bacteria. The type I systems from *E. coli* and *Salmonella,* as well as the type II system from *S. thermophilus* were also used to selectively eliminate even closely related organisms (99% genomic identity) by targeting unique sequences in a complex microbial population [29]. Across types and subtypes and organisms, investigators have generally observed that self-targeting using endogenous CRISPR-Cas systems in bacteria kills the large majority of the bacterial population, with killing efficiency ranging between 2 and 5 orders of magnitude of cell death, with single spacers.

Causing DNA damage with Cas nucleases

Most organisms, including bacteria, are regularly subjected to double stranded breaks and other types of DNA damage. Consequently, they have evolved complex DNA repair pathways that enable to maintain genomic integrity. One might thus wonder why CRISPR-Cas systems are so efficient at killing bacteria. In the case of type I systems, Cas3 possesses both ssDNA exonuclease and 3' to 5' helicase activities [34,35]. The introduction or activation of a self-targeting type I system leads to immediate cell death and efficient degradation of DNA up to 100kb away from the target position in a few hours [32]. In type II systems however, Cas9 only introduces a double strand break (DSB). DSBs are recognized by exonucleases such as recBCD or addAB, which create ssDNA substrates for homology directed repair, via homologous recombination [36]. The consequences of Cas9 cleavage in the chromosome of *E. coli* was recently investigated in detail [37]. It was found that some crRNAs efficiently guide Cas9 to cut all copies of the chromosome simultaneously, making repair through recombination with a sister chromosome impossible and thus efficiently killing the cell. However, other crRNAs lead to less efficient targeting resulting in cell survival thanks to a continuous loop of cleavage and repair. The authors were able to block this phenomenon by expressing the Gam protein from bacteriophage Mu during Cas9 targeting. This protein binds to double stranded ends and blocks homologous recombination, leading to cell death regardless of the target choice. By controlling the outcome of CRISPR self-targeting, scientists can thus either edit genomes or induce cell death.

Delivery

While CRISPR self-targeting has been shown in principle to be a potent programmable antimicrobial, the main challenge for its repurposing to eradicate bacteria responsible for infectious disease is delivery to the target population, with both specificity and efficiency that would afford clinically-

relevant efficacy. Several studies have shown how DNA encoding bactericidal proteins other than Cas nucleases can be delivered to bacterial populations using phage particles as vectors. The M13 phagemid was used to deliver various toxins or restriction enzymes to *E. coli* [38–40]. The Pf3 phage has also been used to a deliver a restriction enzyme, and successful treat a *P. aeruginosa* infection in mice [41].

Inspired by these early results, two studies, one in *E. coli*, the other in *S. aureus*, have provided a proof of concept for delivery of self-targeting CRISPR-Cas systems to pathogenic bacteria using phage capsids as delivery vectors [12,42]. In the first study, the M13 phagemid system was used to inject a genetic construct containing Cas9 and guide RNAs targeting various antibiotic resistance genes, namely bla NDM-1, blaSHV-18, and gyrAD87G. As expected, when the target gene was present in the chromosome, the outcome of Cas9 cleavage was efficient *E. coli* cell death. Interestingly, targeting a plasmid could also lead to cell death when the plasmid carried a toxin-antitoxin system, but otherwise yielded plasmid curing. In the second study, the authors constructed a phagemid based on phage phiNM1 by cloning its packaging site on a plasmid carrying the CRISPR-Cas system, to target *S. aureus*. This phagemid was used to target various antibiotic resistance genes, as well as virulence factors carried either on plasmids or on the chromosome. Both studies demonstrated the possibility of using CRISPR-Cas systems to specifically eliminate a target bacterial genotype in a mixed population, both *in vitro* and *in vivo*, using a wax worm infection model in the first case and a mouse skin colonization model in the other.

Delivering the CRISPR-Cas system to the majority of a target population in a complex environment, where the disease agent might be present in only small amounts, represents a big challenge. Qimron and colleagues proposed an elegant strategy to give a fitness advantage to bacteria that receive the CRISPR-Cas system [43]. In a first step they delivered a CRISPR-Cas system carrying a set of spacers targeting beta-lactam resistance genes. This presumably re-sensitizes *E. coli* cells that carry plasmids with the targeted sequences to the antibiotic. In a second step, they selected bacteria that received this system by using a lytic T7 phage modified to carry target sequences matching the CRISPR spacers. Only cells that carry an active CRISPR-Cas systems with the proper anti-beta lactam spacers can resist infection by the modified T7 phage. This strategy enables the genesis of a population of bacteria that are all re-sensitized and that carry the CRISPR-Cas system. Nevertheless, it may not prove useful in cases where the targets are present in the chromosome or on plasmids carrying toxin-antitoxin systems.

One key consideration for engineering moving forward is assessing which class, type and subtype of CRISPR-Cas systems are most lethal and effective, and determining whether it is preferable to co-opt

endogenous systems and "just" deliver multiplexed self-targeting CRISPR arrays, or co-deliver the CRISPR targeting array and the corresponding effector Cas machinery (Figure 1b). Delivering a CRISPR array alone presents the advantage of requiring a smaller and simpler construct, but the drawback of relying on endogenous Cas proteins which might not be expressed in all conditions in the recipient strains [44]. Once the CRISPR machinery is readily packaged into proper viral vectors, the optimal formulation of bacteriophage preparation will have to be developed and optimized, potentially differentially for each delivery route. Indeed, delivery format is anticipated to vary together with the target organism and site of infection (i.e. intestinal vs. topical vs. lung infections vs. urogenital tract).

Resistance

An important limitation of all strategies that employ phages as delivery vectors is their host range. Most phages can only infect a limited number of strains within a given species. Host range can be limited by a number of factors [45]. Surface receptors might not be present or can be hidden; entry exclusion systems can block DNA injection in the cell; restriction-modification systems and CRISPR-Cas systems can degrade the phage DNA; and finally, abortive infection systems can block later stages of the phage cycle by coercing infected cell to commit suicide. With the exception of abortive infection systems which would not be triggered by a synthetic genetic circuit, all these phage defense pathways can also present obstacles to the delivery of CRISPR DNA into bacteria (Figure 1). Phage cocktails have traditionally been used to overcome these hurdles by ensuring that at least one phage is able to infect the target bacteria, but these cocktails can be complex to formulate and produce at industrial scales, and also present additional regulatory challenges [46]. Phages with extended host ranges can also be obtained either through engineering or selection [47,48]. In a recent study Qimron and colleagues demonstrate how a T7 phagemid can be used to effectively evolve tails enabling efficient transduction in desired host strains [49].

In addition to resistance mechanisms that block the proper delivery of the CRISPR-Cas system, the CRISPR-Cas system itself can fail in several ways. Mutations in the CRISPR-Cas system or in the target can allow bacteria to survive. In the first case, bacteria would still be sensitive to a functional CRISPR-Cas system, but in the second case, they would effectively escape. Several studies have shown how large deletions can occur in the target region enabling bacteria to survive [30,37,50]. Such outcomes can be seen as positive, as they would result in the loss of antibiotic resistance or virulence traits. Nonetheless, point mutations that preserve the gene function might enable bacteria to escape recognition by the Cas nucleases. This can be solved easily by programming the CRISPR-Cas system to target several positions at the undesired locus simultaneously. Fittingly, CRISPR are by nature arrays that encompass multiple targeting sequences, enabling easy and convenient multiplexing for

antimicrobials. Finally, anti-CRISPR proteins have recently been characterized in some phages [51]. In future, they might be coopted by bacteria to escape CRISPR antimicrobial therapies.

Perspectives

CRISPR-Cas systems have been successfully repurposed to target virulence factors and antibiotic resistance genes in bacteria, and constitute an appealing option for programmable and sequence-specific antimicrobials. They can efficiently kill a target population when delivered by phage capsids *in vitro* and can also reduce the colonization of a target population *in vivo*. In addition, resistant bacteria can be re-sensitized to an antibiotic by curing plasmids carrying resistance genes. In order to bring these strategies to the clinic, specific indications and therapeutic approaches will have to be established. These therapeutic approaches should also be discussed in comparison to other specific antimicrobials such as phage therapy, antimicrobial peptides, antibodies or vaccines, which was not possible within the framework of this short review. The unique advantage of CRISPR-based antimicrobials over all these other strategies is their ability to kill bacteria based on their sequence. This might prove advantageous in cases where it is desirable to eliminate only a select group of bacteria within a species, something that would be arguably impossible to achieve with incumbent strategies. Of course, CRISPR-based approaches would also address two grand challenges of currently available antibiotics, namely: (1) to prevent the indiscriminate eradication of bacteria that might be beneficial; (2) to lessen the selective pressure for resistance (by allowing the non-target population to thrive and occupy the ecological niche). This opens new avenues for CRISPR-based technologies to control the composition of microbial communities rather than using it as a traditional broad-spectrum antibiotic.

Financial and competing interests disclosure

The authors are inventors on several patents related to various uses of CRISPR-based technologies. RB is a co-founder and SAB member of Intellia Therapeutics and Locus Biosciences. DB is co-founder and SAB member of Eligo Bioscience.

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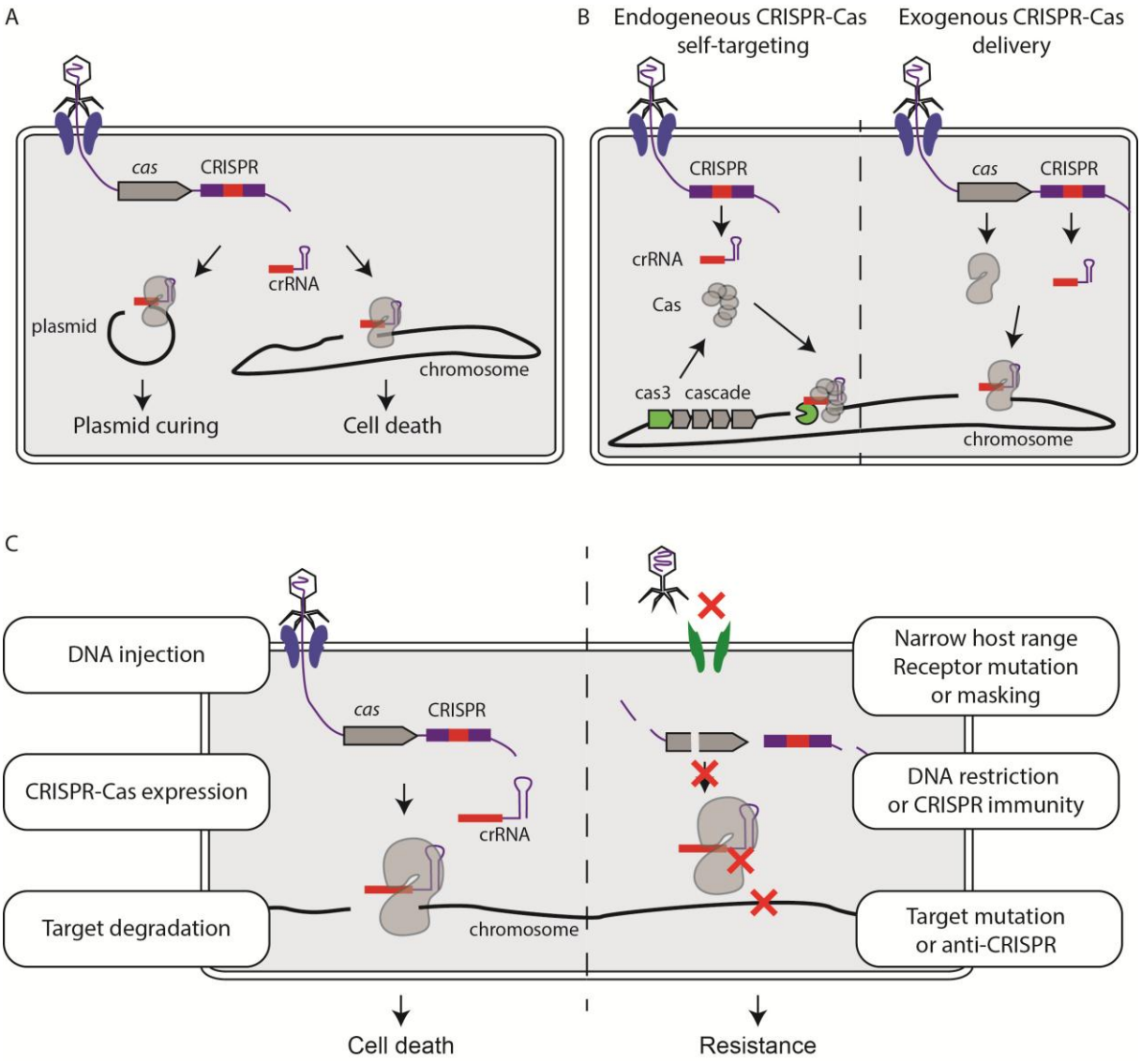

Figure 1. CRISPR antimicrobials. A) After injection of a CRISPR system, Cas nuclease cleavage of a target carried by a plasmid leads to plasmid loss while cleavage in the chromosome leads to cell death. B) If the target bacterium carries a endogenous CRISPR-Cas system, one can simply deliver a self-targeting CRISPR array to direct Cas nucleases towards a desired locus. Another strategy is to deliver an exogenous CRISPR-Cas system. C) Summary of CRISPR antimicrobials action and possible resistance mechanisms at every step. The phage vector might not be able to inject its DNA due to narrow host range, receptor mutations or masking. After injection, DNA can be degraded by the action of restriction enzymes or CRISPR-Cas systems. Finally, anti-CRISPR proteins or mutations in the target sequence might block target recognition and cleavage.

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