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Using CRISPR-Cas systems as antimicrobials

David Bikard¹ & Rodolphe Barrangou²

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

1 **Abstract**

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

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

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

40 **Self-targeting by CRISPR-Cas systems in nature**

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

45 spacers that perfectly match sequences encoded on the host chromosome [19]. While some of these
46 self-targeting spacers match prophages and other mobile elements such as transposons, others are
47 found to match genes surprisingly belonging to the core genome. Nonetheless, in almost all of these
48 cases, the authors were able to identify clues that the CRISPR-Cas system has been somehow
49 inactivated by mutations in the *cas* genes or CRISPR array, or mutations altering the targeted
50 sequences. Mutations in the target sequence that block CRISPR immunity typically occur in the
51 protospacer-adjacent motif (PAM) (Horvath et al., 2008), or within the seed sequence [20,21], which
52 encompasses the cleavage site and drives the formation of the crRNA:targetDNA R-loop for cleavage
53 [22]. A model was thus proposed where the CRISPR-Cas systems sometimes capture self-targeting
54 spacers “by mistake” and can only survive such events if the system is functionally inactivated. A
55 study investigating the spacer acquisition process in the *Streptococcus thermophilus* model system
56 showed that acquisition from chromosomal sequences can occur, but established that this auto-
57 immune sampling is rare, and that when it does occur, these genotypes do not remain in the
58 bacterial population [23]. Specifically, the authors were able to detect acquisition from chromosomal
59 sequences at a frequency of 0.04% (120 of 443,871 acquired spacers), and showed that these self-
60 targeting events could only be detected at one time point, and disappeared within a day from the
61 population, presumably because acquisition of self-targeting spacers is lethal. Several mechanisms
62 enable CRISPR-Cas systems to preferentially acquire spacers from foreign DNA, including primed
63 adaptation [24] and acquisition from DNA fragments generated after the processing by exonucleases
64 of double strand breaks, or phage DNA termini [25,26]. Despite these mechanisms, it is possible to
65 detect lethal acquisition events in natural CRISPR-Cas systems [27].

66 **The primary outcome of self-targeting is cell death**

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

79 the ability to cure plasmids, or kill bacteria when reprogrammed to target the chromosome (Figure
80 1a) [33].

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

90 **Causing DNA damage with Cas nucleases**

91 Most organisms, including bacteria, are regularly subjected to double stranded breaks and other
92 types of DNA damage. Consequently, they have evolved complex DNA repair pathways that enable to
93 maintain genomic integrity. One might thus wonder why CRISPR-Cas systems are so efficient at killing
94 bacteria. In the case of type I systems, Cas3 possesses both ssDNA exonuclease and 3' to 5' helicase
95 activities [34,35]. The introduction or activation of a self-targeting type I system leads to immediate
96 cell death and efficient degradation of DNA up to 100kb away from the target position in a few hours
97 [32]. In type II systems however, Cas9 only introduces a double strand break (DSB). DSBs are
98 recognized by exonucleases such as recBCD or addAB, which create ssDNA substrates for homology
99 directed repair, via homologous recombination [36]. The consequences of Cas9 cleavage in the
100 chromosome of *E. coli* was recently investigated in detail [37]. It was found that some crRNAs
101 efficiently guide Cas9 to cut all copies of the chromosome simultaneously, making repair through
102 recombination with a sister chromosome impossible and thus efficiently killing the cell. However,
103 other crRNAs lead to less efficient targeting resulting in cell survival thanks to a continuous loop of
104 cleavage and repair. The authors were able to block this phenomenon by expressing the Gam protein
105 from bacteriophage Mu during Cas9 targeting. This protein binds to double stranded ends and blocks
106 homologous recombination, leading to cell death regardless of the target choice. By controlling the
107 outcome of CRISPR self-targeting, scientists can thus either edit genomes or induce cell death.

108 **Delivery**

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

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

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

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

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

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

153 **Resistance**

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

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

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

180 **Perspectives**

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

199 **Financial and competing interests disclosure**

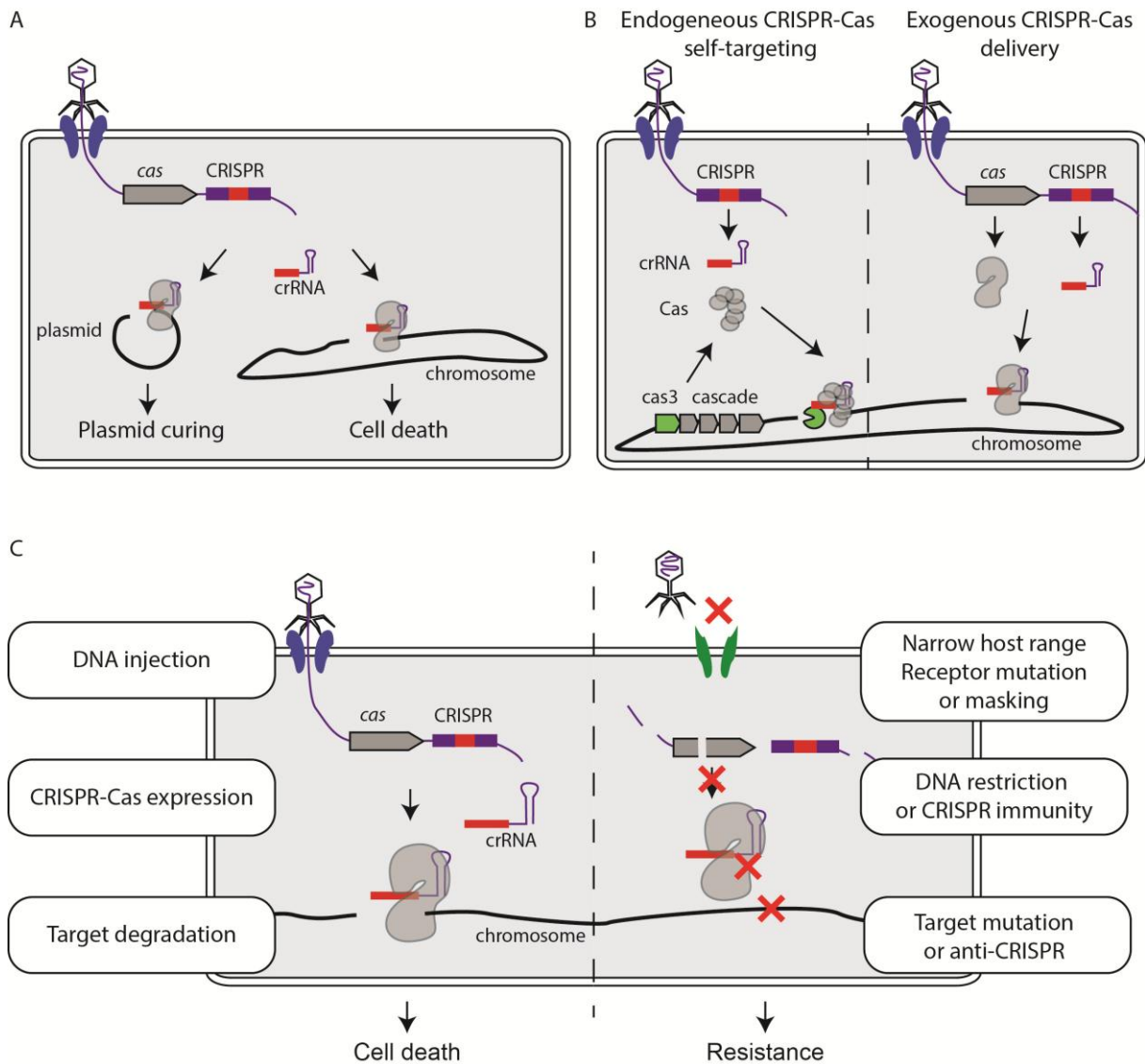
200 The authors are inventors on several patents related to various uses of CRISPR-based technologies.
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212

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

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