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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.

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

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-

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

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.
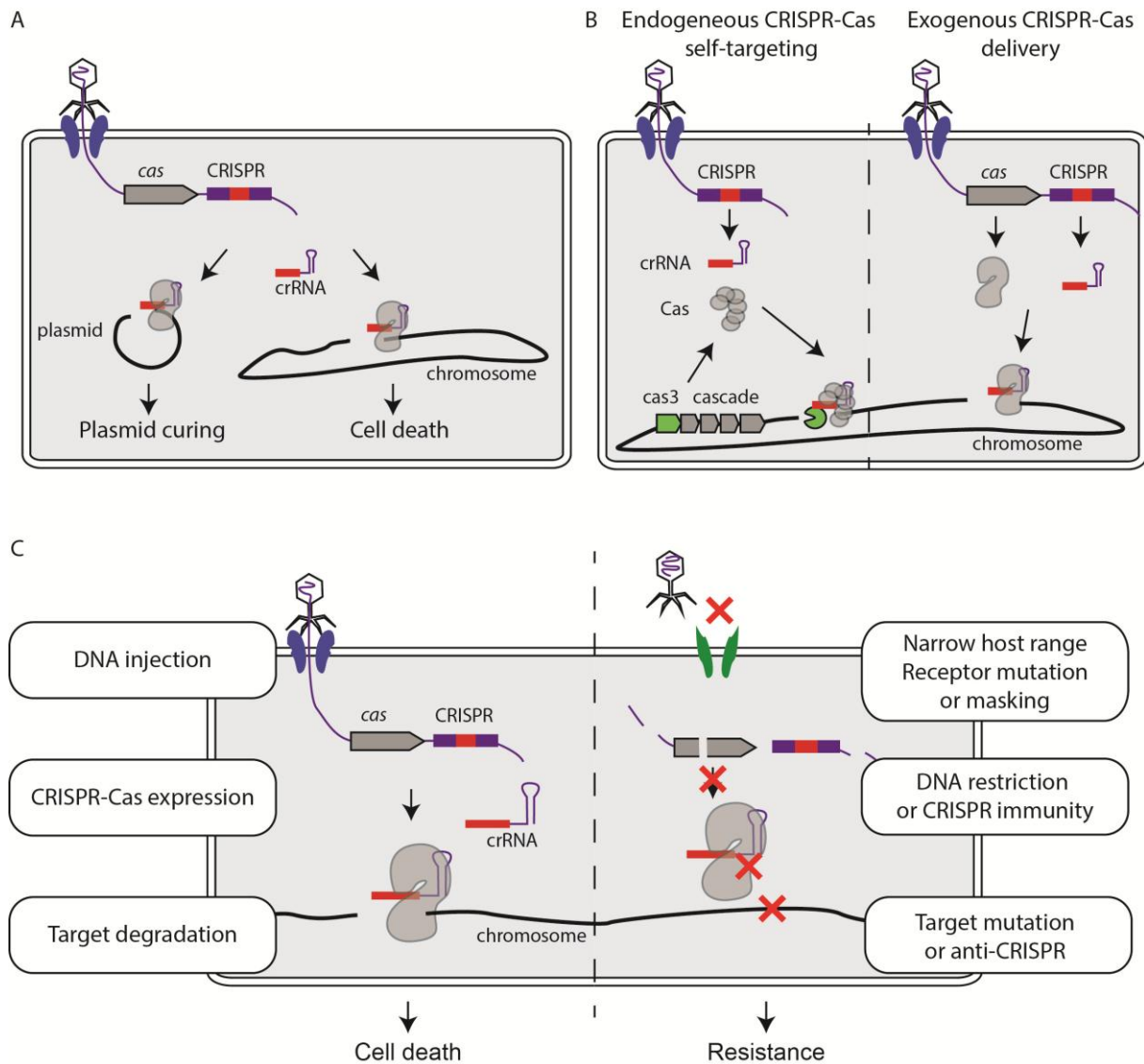
180   **Perspectives**

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.

209

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.

References

222   1.   Marraffini LA: **CRISPR-Cas immunity in prokaryotes**. *Nature* 2015, **526**:55–61.

223   2.   Hsu PD, Lander ES, Zhang F: **Development and applications of CRISPR-Cas9 for genome
224   engineering**. *Cell* 2014, **157**:1262–78.

225   3.   Ledford H: **CRISPR, the disruptor**. *Nat. News* 2015, **522**:20.

226   4.   Pennisi E: **The CRISPR craze**. *Science* 2013, **341**:833–6.

227   5.   Makarova KS, Wolf YI, Alkhnbashi OS, Costa F, Shah SA, Saunders SJ, Barrangou R, Brouns SJJ,
228   Charpentier E, Haft DH, et al.: **An updated evolutionary classification of CRISPR-Cas systems**. *Nat.
229   Rev. Microbiol.* 2015, **13**:722–736.

230   * This study updated the classification and nomenclature of CRISPR-Cas systems and outlined their
231   occurrence and diversity in bacteria and archaea.

232   6.   Brouns SJ, Jore MM, Lundgren M, Westra ER, Slijkhuis RJ, Snijders AP, Dickman MJ, Makarova
233   KS, Koonin EV, van der Oost J: **Small CRISPR RNAs guide antiviral defense in prokaryotes**. *Science*
234   2008, **321**:960–4.

235   7.   Sinkunas T, Gasiunas G, Waghmare SP, Dickman MJ, Barrangou R, Horvath P, Siksnys V: **In
236   vitro reconstitution of Cascade-mediated CRISPR immunity in Streptococcus thermophilus**. *EMBO J*
237   2013, **32**:385–94.

238   8.   Garneau JE, Dupuis ME, Villion M, Romero DA, Barrangou R, Boyaval P, Fremaux C, Horvath P,
239   Magadan AH, Moineau S: **The CRISPR/Cas bacterial immune system cleaves bacteriophage and
240   plasmid DNA**. *Nature* 2010, **468**:67–71.

241   9.   Gasiunas G, Barrangou R, Horvath P, Siksnys V: **Cas9–crRNA ribonucleoprotein complex
242   mediates specific DNA cleavage for adaptive immunity in bacteria**. *Proc. Natl. Acad. Sci.* 2012,
243   **109**:15539–15540.

244   10.   Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E: **A programmable dual-
245   RNA-guided DNA endonuclease in adaptive bacterial immunity**. *Science* 2012, **337**:816–21.

246   11.   Barrangou R, Doudna JA: **Applications of CRISPR technologies in research and beyond**. *Nat.
247   Biotechnol.* 2016, **34**:933–941.

248   12.   Citorik RJ, Mimee M, Lu TK: **Sequence-specific antimicrobials using efficiently delivered
249   RNA-guided nucleases**. *Nat Biotechnol* 2014, **32**:1141–5.

250   ** This report established a proof of concept that CRISPR-Cas9 systems can be delivered by phages to
251   target E. coli in vitro and in vivo.

252   13.   Bikard D, Jiang W, Samai P, Hochschild A, Zhang F, Marraffini LA: **Programmable repression
253   and activation of bacterial gene expression using an engineered CRISPR-Cas system**. *Nucleic Acids
254   Res.* 2013, **41**:7429–7437.

255   14.   Jiang W, Bikard D, Cox D, Zhang F, Marraffini LA: **RNA-guided editing of bacterial genomes
256   using CRISPR-Cas systems**. *Nat Biotechnol* 2013, **31**:233–239.

257   15.   Luo ML, Mullis AS, Leenay RT, Beisel CL: **Repurposing endogenous type I CRISPR-Cas systems**
258   **for programmable gene repression**. *Nucleic Acids Res.* 2015, **43**:674–681.

259   16.   Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP, Lim WA: **Repurposing**
260   **CRISPR as an RNA-guided platform for sequence-specific control of gene expression**. *Cell* 2013,
261   **152**:1173–83.

262   17.   Goren M, Yosef I, Qimron U: **Sensitizing pathogens to antibiotics using the CRISPR-Cas**
263   **system**. *Drug Resist. Updat. Rev. Comment. Antimicrob. Anticancer Chemother.* 2017, **30**:1–6.

264   18.   Horvath P, Romero DA, Coute-Monvoisin AC, Richards M, Deveau H, Moineau S, Boyaval P,
265   Fremaux C, Barrangou R: **Diversity, activity, and evolution of CRISPR loci in Streptococcus**
266   **thermophilus**. *J Bacteriol* 2008, **190**:1401–12.

267   19.   Stern A, Keren L, Wurtzel O, Amitai G, Sorek R: **Self-targeting by CRISPR: gene regulation or**
268   **autoimmunity?** *Trends Genet* 2010, **26**:335–40.

269   20.   Semenova E, Jore MM, Datsenko KA, Semenova A, Westra ER, Wanner B, van der Oost J,
270   Brouns SJ, Severinov K: **Interference by clustered regularly interspaced short palindromic repeat**
271   **(CRISPR) RNA is governed by a seed sequence**. *Proc Natl Acad Sci U A* 2011, **108**:10098–103.

272   21.   Wiedenheft B, van Duijn E, Bultema JB, Waghmare SP, Zhou K, Barendregt A, Westphal W,
273   Heck AJ, Boekema EJ, Dickman MJ, et al.: **RNA-guided complex from a bacterial immune system**
274   **enhances target recognition through seed sequence interactions**. *Proc Natl Acad Sci U A* 2011,
275   **108**:10092–7.

276   22.   Sternberg SH, Redding S, Jinek M, Greene EC, Doudna JA: **DNA interrogation by the CRISPR**
277   **RNA-guided endonuclease Cas9**. *Nature* 2014, **507**:62–7.

278   23.   Paez-Espino D, Morovic W, Sun CL, Thomas BC, Ueda K, Stahl B, Barrangou R, Banfield JF:
279   **Strong bias in the bacterial CRISPR elements that confer immunity to phage**. *Nat. Commun.* 2013,
280   **4**:1430.

281   24.   Datsenko KA, Pougach K, Tikhonov A, Wanner BL, Severinov K, Semenova E: **Molecular**
282   **memory of prior infections activates the CRISPR/Cas adaptive bacterial immunity system**. *Nat*
283   *Commun* 2012, **3**:945.

284   25.   Levy A, Goren MG, Yosef I, Auster O, Manor M, Amitai G, Edgar R, Qimron U, Sorek R: **CRISPR**
285   **adaptation biases explain preference for acquisition of foreign DNA**. *Nature* 2015, **520**:505–510.

286   26.   Modell JW, Jiang W, Marraffini LA: **CRISPR-Cas systems exploit viral DNA injection to**
287   **establish and maintain adaptive immunity**. *Nature* 2017, **544**:101–104.

288   27.   Staals RHJ, Jackson SA, Biswas A, Brouns SJJ, Brown CM, Fineran PC: **Interference-driven**
289   **spacer acquisition is dominant over naive and primed adaptation in a native CRISPR-Cas system**.
290   *Nat. Commun.* 2016, **7**:12853.

291   28.   Edgar R, Qimron U: **The Escherichia coli CRISPR system protects from lambda**
292   **lysogenization, lysogens, and prophage induction**. *J Bacteriol* 2010, **192**:6291–4.

293   * This is the first report of artificial self targeting by a CRISPR system

294   29.   Gomaa AA, Klumpe HE, Luo ML, Selle K, Barrangou R, Beisel CL: **Programmable removal of**
295   **bacterial strains by use of genome-targeting CRISPR-Cas systems**. *MBio* 2013, **5**:e00928-13.

296   ** This study showed that endogenous CRISPR-Cas systems can be repurposed to generate
297   programmable cell death in bacteria and selectively target different genotypes in a mixed population.

298   30.   Vercoe RB, Chang JT, Dy RL, Taylor C, Gristwood T, Clulow JS, Richter C, Przybilski R, Pitman
299   AR, Fineran PC: **Cytotoxic chromosomal targeting by CRISPR/Cas systems can reshape bacterial**
300   **genomes and expel or remodel pathogenicity islands**. *PLoS Genet* 2013, **9**:e1003454.

301   * This early report revealed the lethality and some genomic outcomes of self-targeting by CRISPR-Cas
302   systems.

303   31.   Westra ER, van Erp PB, Kunne T, Wong SP, Staals RH, Seegers CL, Bollen S, Jore MM,
304   Semenova E, Severinov K, et al.: **CRISPR immunity relies on the consecutive binding and**
305   **degradation of negatively supercoiled invader DNA by Cascade and Cas3**. *Mol Cell* 2012, **46**:595–
306   605.

307   32.   Caliando BJ, Voigt CA: **Targeted DNA degradation using a CRISPR device stably carried in the**
308   **host genome**. *Nat. Commun.* 2015, **6**:6989.

309   33.   Bikard D, Hatoum-Aslan A, Mucida D, Marraffini LA: **CRISPR interference can prevent natural**
310   **transformation and virulence acquisition during in vivo bacterial infection**. *Cell Host Microbe* 2012,
311   **12**:177–186.

312   34.   Beloglazova N, Petit P, Flick R, Brown G, Savchenko A, Yakunin AF: **Structure and activity of**
313   **the Cas3 HD nuclease MJ0384, an effector enzyme of the CRISPR interference**. *EMBO J.* 2011,
314   **30**:4616–4627.

315   35.   Sinkunas T, Gasiunas G, Fremaux C, Barrangou R, Horvath P, Siksnys V: **Cas3 is a single-**
316   **stranded DNA nuclease and ATP-dependent helicase in the CRISPR/Cas immune system**. *EMBO J*
317   2011, **30**:1335–42.

318   36.   Wigley DB: **Bacterial DNA repair: recent insights into the mechanism of RecBCD, AddAB and**
319   **AdnAB**. *Nat. Rev. Microbiol.* 2013, **11**:9–13.

320   37.   Cui L, Bikard D: **Consequences of Cas9 cleavage in the chromosome of Escherichia coli**.
321   *Nucleic Acids Res.* 2016, **44**:4243–4251.

322   * This study investigated the outcomes of Cas9-based self-targeting in bacteria and established the
323   interplay between DNA damage and DNA repair.

324   38.   Hagens S, Bläsi U: **Genetically modified filamentous phage as bactericidal agents: a pilot**
325   **study**. *Lett. Appl. Microbiol.* 2003, **37**:318–323.

326   39.   Moradpour Z, Sepehrizadeh Z, Rahbarizadeh F, Ghasemian A, Yazdi MT, Shahverdi AR:
327   **Genetically engineered phage harbouring the lethal catabolite gene activator protein gene with an**
328   **inducer-independent promoter for biocontrol of Escherichia coli**. *FEMS Microbiol. Lett.* 2009,
329   **296**:67–71.

330   40.   Westwater C, Kasman LM, Schofield DA, Werner PA, Dolan JW, Schmidt MG, Norris JS: **Use of**
331   **Genetically Engineered Phage To Deliver Antimicrobial Agents to Bacteria: an Alternative Therapy**
332   **for Treatment of Bacterial Infections**. *Antimicrob. Agents Chemother.* 2003, **47**:1301–1307.

333 41. Hagens S, Habel A, Ahsen U von, Gabain A von, Bläsi U: **Therapy of Experimental**
334 **Pseudomonas Infections with a Nonreplicating Genetically Modified Phage**. *Antimicrob. Agents*
335 *Chemother.* 2004, **48**:3817–3822.

336 42. Bikard D, Euler CW, Jiang W, Nussenzweig PM, Goldberg GW, Duportet X, Fischetti VA,
337 Marraffini LA: **Exploiting CRISPR-Cas nucleases to produce sequence-specific antimicrobials**. *Nat*
338 *Biotechnol* 2014, **32**:1146–50.

339 ** This report established a proof of concept that CRISPR-Cas9 systems can be delivered by phages to
340 target S. aureus in vitro and in vivo.

341 43. Yosef I, Manor M, Kiro R, Qimron U: **Temperate and lytic bacteriophages programmed to**
342 **sensitize and kill antibiotic-resistant bacteria**. *Proc. Natl. Acad. Sci.* 2015, **112**:7267–7272.

343 ** This study provides novel strategies to re-sensitize bacteria to antibiotics using plasmid targeting
344 CRISPR-Cas systems.

345 44. Westra ER, Pul Ü, Heidrich N, Jore MM, Lundgren M, Stratmann T, Wurm R, Raine A, Mescher
346 M, Van Heereveld L, et al.: **H-NS-mediated repression of CRISPR-based immunity in Escherichia coli**
347 **K12 can be relieved by the transcription activator LeuO**. *Mol. Microbiol.* 2010, **77**:1380–1393.

348 45. Labrie SJ, Samson JE, Moineau S: **Bacteriophage resistance mechanisms**. *Nat Rev Microbiol*
349 2010, **8**:317–27.

350 46. Sulakvelidze A, Alavidze Z, Morris JG: **Bacteriophage Therapy**. *Antimicrob. Agents Chemother.*
351 2001, **45**:649–659.

352 47. Ando H, Lemire S, Pires DP, Lu TK: **Engineering Modular Viral Scaffolds for Targeted Bacterial**
353 **Population Editing**. *Cell Syst.* 2015, **1**:187–196.

354 48. Mapes AC, Trautner BW, Liao KS, Ramig RF: **Development of expanded host range phage**
355 **active on biofilms of multi-drug resistant Pseudomonas aeruginosa**. *Bacteriophage* 2016,
356 **6**:e1096995.

357 49. Yosef I, Goren MG, Globus R, Molshanski-Mor S, Qimron U: **Extending the Host Range of**
358 **Bacteriophage Particles for DNA Transduction**. *Mol. Cell* 2017, **66**:721–728.e3.

359 50. Selle K, Klaenhammer TR, Barrangou R: **CRISPR-based screening of genomic island excision**
360 **events in bacteria**. *Proc. Natl. Acad. Sci.* 2015, **112**:8076–8081.

361 * This report established that native CRISPR-Cas systems can be co-opted to select for rare deletion
362 events of expendable genetic islands.

363 51. Pawluk A, Staals RHJ, Taylor C, Watson BNJ, Saha S, Fineran PC, Maxwell KL, Davidson AR:
364 **Inactivation of CRISPR-Cas systems by anti-CRISPR proteins in diverse bacterial species**. *Nat.*
365 *Microbiol.* 2016, **1**:16085.

366