

# CRISPR-Cas Systems: A Review of their Role as Non-Canonical Antimicrobials to Combat Drug Resistance

Oluwafolajimi Adetoye Adesanya<sup>1\*</sup> and Segun Abayomi Oniyide<sup>2</sup>

<sup>1</sup>*Institute for Advanced Medical Research and Training (IAMRAT), College of Medicine, University of Ibadan, Ibadan, Nigeria.*

<sup>2</sup>*Department of Medicine, College of Medicine, University of Ibadan, Ibadan, Nigeria.*

## **Authors' contributions**

*This work was carried out in collaboration between both authors. Author OAA conceived the study, search and reviewed the literature and drafted the manuscript. Author SAO reviewed the manuscript and provided the images and illustrations. Both authors have read and approved the final manuscript for publication.*

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## **ABSTRACT**

**Background:** Since their discovery in 1928, antibiotics have revolutionized modern human medicine. A direct consequence of their widespread use and abuse is the concomitant development of sophisticated resistance mechanisms by microbes to most of these antibiotic agents. With a sharp fall in the number of new antibiotic agents under development, due to a decline in sectoral investment by the major pharmaceutical firms, now more than ever, there is an urgent need for alternative antimicrobial agents, to overcome the global health crisis of antimicrobial resistance. One such alternative are the CRISPR-Cas systems. In this review, we discuss the discovery and evolution of the CRISPR-Cas system, their classification, their application as antimicrobial agents and the current limitations to their use.

**Main Body:** Since their discovery in 1987, the CRISPR-Cas systems have evolved to possess several applications in biomedical science, including their use in gene editing, genome engineering and for treating genetic diseases. One potential application that has received less attention however

\*Corresponding author: E-mail: folajimiadesanya@gmail.com;

is their use as sequence-specific antimicrobials. The three steps in their mechanism of action – spacer acquisition, biogenesis and interference, have been demonstrated to be effective for the highly-specific elimination of targeted bacterial strains from a mixed population of bacteria in a microbial community, as well as for driving a positive selection pressure for the development of antibiotic-sensitive bacterial strains, even to the point of reversing antimicrobial resistance through the elimination of the resistance genes. However, the use of CRISPR-based antimicrobials is fraught with several challenges, ranging from concerns about delivery platforms (phage-based vs. conjugative plasmids), their application on complex microbial communities, safety considerations, to the possibility of resistance and the many regulatory hurdles against their widespread use.

**Conclusion:** While their efficacy has been confirmed, more studies are required to further establish CRISPR-Cas based antimicrobials in conventional medical practice.

*Keywords: CRISPR-Cas; antimicrobial resistance; CASCADE; gene editing.*

## ABBREVIATIONS

AMR	: Anti-microbial Resistance
CDC	: United States Centers for Diseases Control and Prevention
CRISPR-Cas	: Clustered Regularly Interspersed Short Palindromic Repeats/CRISPR associated systems
Cas	: CRISPR associated systems
DNA	: Deoxyribonucleic Acid
RNA	: Ribonucleic Acid
MGEs	: Mobile Genetic Elements
crRNA	: CRISPR RNA
RNAP	: RNA polymerase
CASCADE	: CRISPR associated complex for antiviral defense
tracrRNA	: Trans-activating crRNA
RAMP	: Repeat Associated Mysterious Protein
qPCR	: Quantitative Polymerase Chain Reaction
acr	: Anti-CRISPR gene
Acr	: Anti-CRISPR protein
FDA	: Food and Drug Administration

## 1. INTRODUCTION: THE RISE OF ANTIMICROBIAL RESISTANCE

Since Fleming's 1928 discovery of penicillin and description of its antibacterial effects, antibiotics have effectively revolutionized the face of modern human medicine as we know it. As a result of their application in clinical practice, bacterial infections which used to be lethal to human health became just another minor spell of ill health, to resolve after a few courses of antibiotics. Their ubiquitous use in clinical medicine is further highlighted by the 262.5 million courses of antibiotics prescribed in the United States in 2011, equivalent to an estimated 842 prescriptions per 1000 persons, and between 100,000 and 200,000 tones of antibiotics being used globally in medicine, agriculture and horticulture [1]. A direct consequence of this widespread use and abuse of antibiotics is their abundant flow into the environment and increased contact time between

these chemicals and the organisms they are designed to kill [2]. This exposure eventually drives the positive selection pressure for microbial strains that have evolved diverse, sophisticated mechanisms for resisting the effects of the known antimicrobial agents [3], some of which include: molecular efflux pumps to eliminate intracellularly acting antibiotics, enzymatic deactivation and mutations of cell surface receptors and proteins [4]. Consequentially, antimicrobial resistance comes at a significant cost, to life and healthy living, with the United States Centers for Disease Control & Prevention (CDC) estimating that antibiotic-resistant infections are responsible for 2 million illnesses and at least 23,000 deaths annually, creating more than \$55 billion in healthcare costs [5]. Another report has predicted that drug-resistant infections will be responsible for 10 million deaths annually by 2050, generating a direct cost of \$100 trillion, in the same time frame [5]. A factor that further exacerbates the drug-

resistance crisis is the sharp decline in the development of novel antibiotic agents, due to a lack of motivation by the major pharmaceutical firms to continue investing time and resources in this space [6]. This low motivation is fuelled by several factors, ranging from: the high cost of research and development of new antibiotic drugs, to the relatively low price charged per treatment, which makes antibiotics development of little economic advantage, when compared with development of other medications such as: cancer chemotherapy drugs and drugs for other chronic pathological conditions [6]. As of June 2019, there were approximately 42 new antibiotics undergoing clinical trials in the United States, according to the Pew Charitable Trust [7], and only 1 in 5 new infectious diseases drugs that enter clinical trials will most likely be approved for patients use [8], underpinning the fact that we need new and alternative therapeutic agents for AMR pathogens. Some of such alternatives currently been explored include bacteriophage therapy, novel antibacterial peptides, nucleic acid based antibacterials, bacteriocins, antibodies and anti-virulence compounds [9]. The limitations observed with antibiotics such as: their broad spectrum of activity and the existential possibility of drug resistance have established some basic criteria which any alternative therapy should fulfill, which include: programmable specificity to target only pathogens of interest, while being ineffective against organisms of the normal microbiome, as well as their rapid adaptability, and ability to target the same species through different mechanisms, thus reducing drastically, the possibility of developing resistance [10]. In this review, we discuss one such alternative, based on the premise that a natural adaptive immune system found in bacterial species can be reprogrammed to act as a lethal agent for microbial killing - the Clustered Regularly Interspersed Short Palindromic Repeats-CRISPR associated system (CRISPR-Cas).

## 2. THE DISCOVERY, EVOLUTION AND MECHANISM OF THE CRISPR-Cas SYSTEM

The discovery of CRISPR-Cas system dates back to 1987, when Ishino et al. reported the discovery of a set of 29 repeating nucleotide (nt) sequences in *E. coli*, while studying the *iap* gene [11]. Over the next decades, further genomic sequencing of bacterial and archaeal strains, revealed more of these repeating nucleotide units, which were initially called clustered repeat

elements, but later named CRISPR by Mojica and colleagues in 2002 [12]. Two major breakthroughs came in 2005 when Mojica et al. [13] as well as Pourcel et al. [14] provided evidence that the repeating units of nucleotide sequences seen in CRISPR loci were indeed derived from extrachromosomal or foreign genetic material. By 2007, Barrangou et al. [15] elucidated the full structure and mechanism of action of the CRISPR-Cas system and in a landmark paper in 2012, Doudna et al. [16] reported the potential application of the CRISPR-Cas system for genome editing. Fig. 1 highlights the major milestones in the development and evolution of the CRISPR-Cas system. CRISPR-Cas is an adaptive immune system found in bacteria and archae [15] and consists of a genetic CRISPR locus, made up of two components: 'spacers' and 'repeats'. While repeats are repetitive sections, consisting of 6-20 genes arranged *in tandem* and encoding the CRISPR-associated (Cas) proteins [17,18], spacers are non-repetitive sections of short nucleotide sequences derived from foreign DNA or RNA such as: bacteriophages, conjugative plasmids and other mobile genetic elements (MGEs) [19], which flank the repeats on both sides, forming an array. The CRISPR-Cas system degrades foreign genetic materials in three steps (Fig. 2), namely: spacer acquisition (or adaptation), biogenesis (or expression of CRISPR RNA) and Interference [20]. Spacer acquisition is the process by which short spacer sequences from the invading genetic material are recognized and integrated into the CRISPR array. The sections of the invading genetic component from which the spacers are acquired are known as: 'protospacers'. This is followed by the synthesis of the CRISPR RNA (crRNA) by the transcription of pre-crRNA into crRNA by RNA polymerase (RNAP). Based on their function of guiding the Cas protein to the target DNA sequence to be cleaved, the crRNA are also known as: guide RNA [21]. Interference is the process whereby the crRNA basepairs perfectly align with the protospacer on the foreign DNA or RNA, and directs the Cas protein to cleave the genetic material on both sides of the pairing [17,18,22]. Consequently, we would expect that mutations in the protospacer adjacent motif (PAM) or any other factor that would lead to a mismatch between the protospacer and the crRNA would render the CRISPR-Cas system ineffective, and make the bacterial cell susceptible to invasion [23]. In addition, we also see that theoretically, this system can be deployed to edit genetic constituents of any cell,

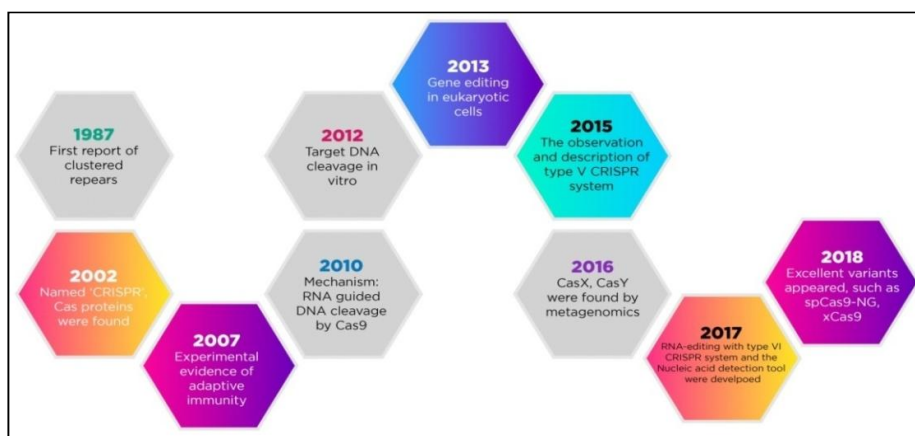


Fig. 1. Evolution of CRISPR-Cas systems

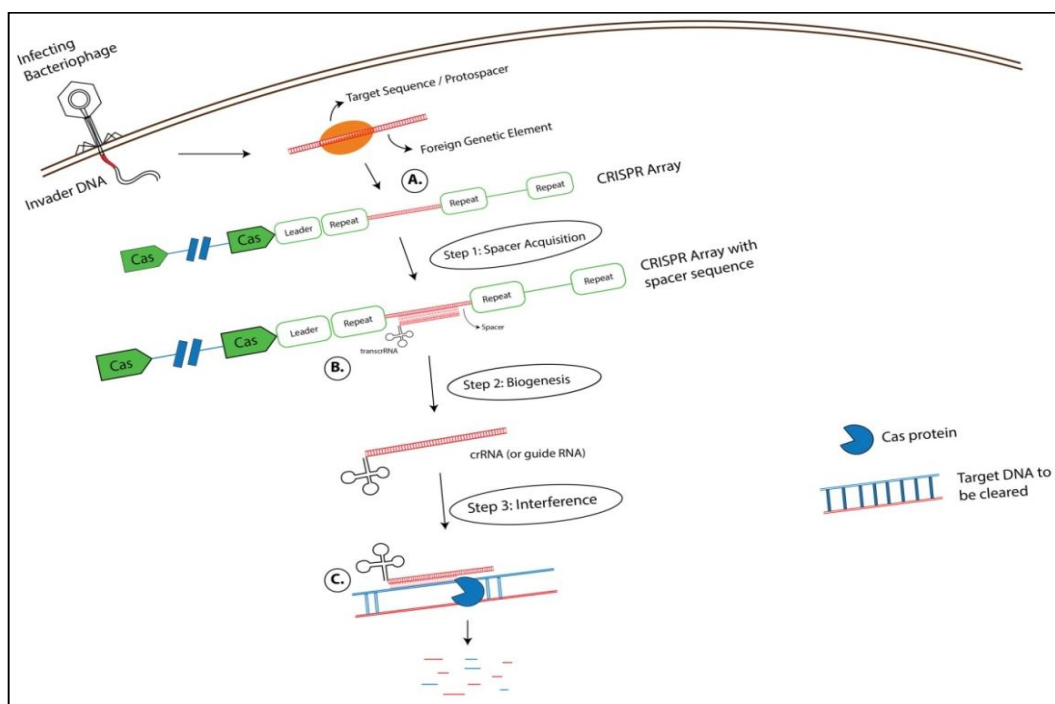


Fig. 2. The CRISPR-Cas system degrades foreign genetic materials in three steps. (A) Target sequence from foreign genetic material is incorporated into the leader end of the CRISPR array on the host cell DNA in the first step known as: Spacer Acquisition. (B) Spacer sequence is transcribed to form the CRISPR-RNA (crRNA) in the second step known as: Biogenesis. (C) crRNA guides the Cas protein to direct cleavage of target sequence on the invading foreign genetic material in the last step known as: Interference

based on the information encoded on the CRISPR array, and subsequently, the crRNA. Till date, the CRISPR-Cas system has been used to edit the genetic content of cells from: humans [24,25], mice [26,27], zebra fish [28], *Drosophila* [29], *C. elegans* [30], plants [31,32], *Saccharomyces* [33], bacteria [34] and bacteriophages [35,36].

### 3. CLASSIFICATION OF THE CRISPR-Cas SYSTEMS

The CRISPR-Cas systems are divided into two classes and six types [37]. Class 1 (consisting of types I, II and IV) are characterized by a complex structure involving multiple Cas proteins which participate in the processes of spacer acquisition

and interference, while class 2 systems (consisting of types II, V and VI) have a simpler structure, in which spacer acquisition and interference are carried out by single, multidomain proteins [37]. The infamous CRISPR-Cas9 system which has been widely deployed for use in genome editing and bioengineering techniques, due to its specific targeting, simplicity and versatility, belongs to the type II class. For the sake of this review, we would be focusing on the Type I (characterized by Cas3), Type II (characterized by Cas9) and Type III (characterized by Cas10) systems. It is important to highlight however that all types and subtypes of CRISPR –Cas systems possess the Cas1 and Cas2 proteins, which are instrumental in the spacer recognition/acquisition process [17,18]. Table 1 highlights the different bacteria within which each of the types is present.

### 3.1 Type I CRISPR-Cas System

The CRISPR-Cas type I system is found in most bacteria and archae and is sub-divided into six sub-types (A-F), all of which encode the Cas3 gene [20]. The Cas3 protein which all members of this sub-type use for cleaving foreign genetic material is a multidomain protein with both nuclease and helicase activity [38]. It contains an N-terminal HD phosphohydrolase domain, which it uses for cleavage and a C-terminal Superfamily 2 (SF2) helicase domain, which it uses to unwind double stranded DNA [17,18,38]. Cas 3 however does not work alone, especially for foreign DNA recognition and spacer acquisition. In each of the subtypes A-F, there are a number of other Cas proteins, which assemble together to form a complex known as the: crRNA guided surveillance complex or the CRISPR associated complex for antiviral defense (CASCADE), which is responsible for foreign DNA/RNA recognition and spacer acquisition, as well as directing the crRNA to bind with its complimentary protospacer [39]. The CASCADE was first

described in *E. coli* K12 (type I-E) [38], and similar complexes have been described in *S. solfataricus* [40], *Pseudomonas aeruginosa* (type I-F) [41] and *Bacillus halodurans* (type I-C) [42].

### 3.2 Type II CRISPR-Cas System

The type II CRISPR-Cas system is found only in bacteria, and is the simplest of the six types, consisting of two sub-types: type II-A and type II-B [37,43]. The type II system is comprised of four genes: Cas1, Cas2, Cas9 and Cas4 (type II-B) or *csn2* (type II-A). The characteristic protein of this type is Cas9, which plays an important role in the crRNA biogenesis and interference stages [44]. In their mechanism of action, the mature crRNA of the type II system binds to a trans-activating crRNA (tracr RNA) to form a two-RNA structure, which then directs the Cas9 protein to cause double-stranded (ds) breaks in the target DNA [16]. The Cas9 protein has a HNH nuclease domain, which cleaves the complimentary strand and a RuvC-like domain, which cleaves the non-complimentary strand [16]. Because all domains needed for DNA cleavage are fused into a single protein molecule – Cas9, the type II systems are widely used in genomic engineering, for manipulation of genetic material.

### 3.3 Type III CRISPR-Cas System

The type III system is most commonly found in archae, and is sub-divided into the type III-A and type III-B [43]. The system is comprised of two main proteins – Cas6 and Cas10. Cas10 is also known as the: repeat associated mysterious protein (RAMP) and is involved in crRNA maturation and interference, while Cas6 is an endoribonuclease [45], which acts independent of the CASCADE to cause cleavage of foreign RNA [46]. Type III CRISPR-Cas systems are often found in conjunction with other CRISPR types, and although they with some similarities,

**Table 1. Distribution of different CRISPR-Cas systems within bacterial species [20]**

CRISPR-Cas system	Bacterial species
Type I	<i>E. coli</i> , <i>P. aeruginosa</i> , <i>M. xanthus</i> , <i>B. halodurans</i> , <i>C. concisus</i> , <i>C. curves</i> , <i>C. fetus</i> , <i>C. hominis</i> , <i>C. rectus</i> , <i>Y. Pestis</i> , <i>Salmonella sp.</i> , <i>E. amylovoran</i> , <i>P. acne</i>
Type II	<i>S. thermophilus</i> , <i>S. mutans</i> , <i>N. meningitides</i> , <i>C. jejuni</i> , <i>L. pneumophilia</i> , <i>L. monocytogenes</i> , <i>F. novicida</i> , <i>S. pyogenes</i> , <i>S. aureus</i> , <i>M. gallisepticum</i> , <i>E. faecalis</i>
Type III	<i>P. furiosus</i> , <i>S. epidermidis</i> , <i>M. tuberculosis</i>

the two subtypes of the Type III system, seem to target different substrates as reported by Shabbir et al. [20]. While the type III-A system of *S. epidermidis* targets DNA substrate, the type III-B system found in *Pyrococcus furiosus* cleaves RNA substrate [17,18].

#### 4. APPLICATION OF CRISPR-Cas SYSTEMS AS ANTIMICROBIAL AGENTS

The application of CRISPR-Cas systems as antimicrobial agents can be visualized as having two main results: bacterial death or resensitization of previously resistant bacteria. While the former is achieved by deploying the CRISPR-Cas system to target chromosomal genes essential for bacterial survival, the later is achieved by deploying the system against the antibiotic resistant genes either found on the chromosome or within intracellular plasmids. In 2014, Gomaa et al. [47], used the CRISPR-Cas system to target specific nucleotide sequences in the genomes of different bacterial strains, an effort which resulted in the death of the targeted strains. To achieve this, they engineered spacer sequences derived from the genome of the targeted bacteria, into the CRISPR-Cas9 array, which they then deployed into a mixed population of bacterial species. They reported that the system was able to differentiate and kill only those bacterial strains which carried sequences complimentary to those of the spacer sequences used, while those lacking these complements were unaffected [47]. This study revealed that the CRISPR-Cas system was indeed an effective programmable, and highly specific antimicrobial agent. The study however had a significant flaw, because they targeted chromosomal genes which were essential for the survival of the bacterial species, rather than extrachromosomal antibiotic resistant genes. Thus, they achieved bacterial killing but at a significant cost. The death of the targeted bacterial species within a population of several mixed bacteria, they created a selection pressure for the non-targeted strains to become resistant to the CRISPR-Cas system used, a situation that would have been avoided, had they targeted extra-chromosomal genes (except if an antibiotic agent is used simultaneously) [48]. In the same year, two sets of authors simultaneously described their experiments in which they used the CRISPR-Cas system to target specific antibiotic resistant genes in bacterial strains. Citorik and colleagues [49], employed two different methods to deliver the CRISPR-Cas system into the target bacteria,

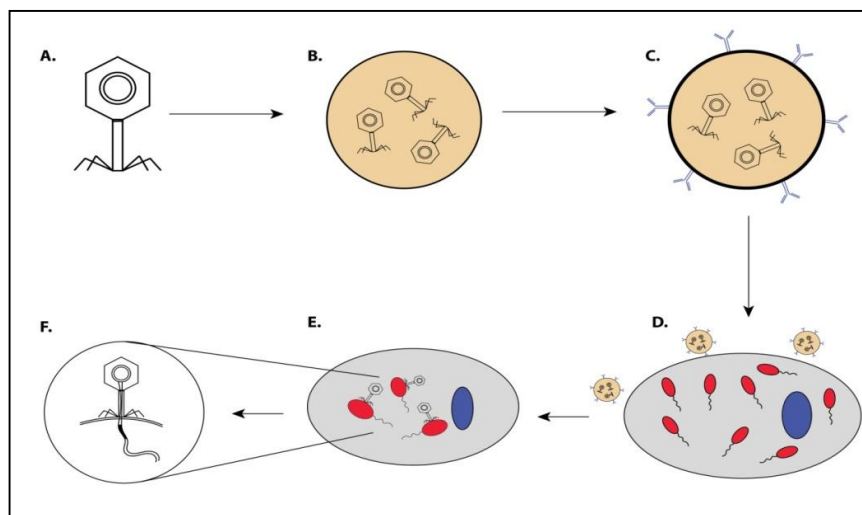
however, unlike Gomaa et al. they were targeting the specific antibiotic resistant genes – *bla*<sub>NDM-1</sub>, *bla*<sub>SHV-18</sub> and *gyr*<sub>AD87G</sub>. The first method involved the use of a conjugative plasmid carried on an *E. coli* strain, such that whenever the target bacteria came in contact with this *E. coli*, the plasmid was exchanged between them by the process of conjugation [49]. This process was however limited by conjugation inefficiencies, necessitating the use of a second delivery technique, which involved an M13 bacteriophage-based phagemid. A phagemid is a phage capsid, used as a delivery vehicle for genetic constituents (this time the CRISPR-Cas system to be used) encoded on its surface. By using the phagemid, they were able to circumvent many of the delivery limitations associated with the conjugation inefficiencies of the conjugative plasmid, as they now rely on the highly efficient and specific system by which the bacteriophage injects its genome into the target bacteria host [50]. By using this phagemid and the CRISPR-Cas system it encoded, to target antibiotic resistant genes in pathogenic *E. coli*, the authors were able to re-establish antibiotic sensitivity in the targeted bacteria strains. They went ahead to test the system in vivo using infected larva, and while they noticed its efficacy in increasing significantly, the survival of the larvae used, compared with the corresponding antibiotic therapy, the CRISPR-Cas system was markedly less potent [49]. Bikard et al. [51], reported a similar study in 2014, in which they used the *Staphylococcus aureus* as target microbe and its bacteriophage  $\Phi$ nm1 as the CRISPR-Cas phagemid delivery platform. By inserting spacers targeting the genes encoding for antibiotic resistance mechanisms in the target *S. aureus* strain, they reported significant resensitization in more than 99% of the bacterial strains in vitro [51]. They further tested their system in skin infected mouse models and noted that the phagemid delivered CRISPR-Cas system significantly reduced the burden of the targeted bacterial strains in the tested mice to a higher extent, when compared with topically administered antibiotic – mupirocin. However, when compared with the systemic antibiotic agent – streptomycin, while the *S. aureus* phagemids failed to eliminate the infection, streptomycin did [51]. Both studies carried out by the Citorik and Bikard groups possessed a significant flaw – they focused on eliminating target bacterial strains from a community of microbial communities, and in so doing, were not able to apply a selection pressure that favors antibiotic sensitivity over antibiotic resistance

[48]. In fact, by targeting only a few strains, they allowed for the others to maintain their antibiotic resistance and even develop resistance mechanisms against the CRISPR-Cas system used. To successfully combat resistance, it is important that a selection pressure opposing that which favors the development of antibiotic resistance be established, rather than simply trying to eliminate the pathogens and the first study to achieve this was by Yosef et al. [52] in 2015. In their work, they were successful in generating a selection pressure against pathogens not sensitized by the CRISPR-Cas system, while making use of the same system to re-sensitize pathogens to antibiotics. To achieve this, they delivered a CRISPR-Cas system designed to cleave antibiotic-resistant genes on intracellular plasmids, while also transferring genes protecting against lysis by virulent phages into the target bacteria using a temperate (non-lytic) bacteriophage. By so doing, they successfully linked antibiotic sensitization to phage protection [52]. When this was then followed by the application of the lytic phage to the microbial community, they were able to generate an artificial selection pressure for the antibiotic-sensitive microbes, and against the resistant ones (which lacked the CRISPR-Cas system that conferred lytic phage protection). Their novel technique was the first of its kind, to

successfully achieve such selective pressure by using a combination of lytic and temperate bacteriophages, and in contrast to the previous endeavors, which focused on killing resistant pathogens; theirs encouraged the emergence of a community of antibiotic sensitive strains [48].

## 5. DELOYING CRISPR-Cas ANTI-MICROBIALS TO COMBAT INTRACELLULAR INFECTIONS

In a situation when the target pathogen is intracellular, as is the case of *Chlamydia trachomatis* and *Burkholderia pseudomallei*, the phagemid encoding the CRISPR-Cas antimicrobial system is faced with a double dilemma, first being its ability to penetrate the host cell to reach the intracellular bacteria, and then to deploy the system successfully into the target pathogen [10]. One possible way to solve this problem is the encapsulation of the phage-encoded CRISPR-Cas system into lipids and silica-based particle structures as demonstrated by Malik et al. [53]. Encapsulated phages, also known as 'bacterial cargoes' can be formulated through a number of processes such as: silica doping or stabilization in protein, and these cargoes allow the phagemid encoding the CRISPR-Cas based antimicrobial system to be delivered safely to the intracellular compartment



**Fig. 3. Intracellular delivery of phage-encoded CRISPR-Cas system. (A) CRISPR-Cas targeting the intracellular pathogen is encoded into the bacteriophage genome. (B) Bacteriophages are encapsulated into silica-based particles known as 'cargoes'. (C) These 'cargoes' are functionalized with a membrane for entry into the target cell. (D) Encapsulated bacteriophage particles are introduced to infected target cell and cell entry takes place. (E) Bacteriophages bind to and attack intracellular pathogen. (F) CRISPR-Cas system is delivered into the intracellular pathogen**

of the infected cell, from where the phageome can be delivered into the target bacteria, for the system to take effect. Fig. 3 summarizes the essential steps involved in the intracellular delivery of phage-encoded CRISPR-Cas systems.

## 6. LIMITATIONS OF CRISPR-Cas BASED ANTIMICROBIALS

### 6.1 Concerning Complex Microbial Populations

So far, most studies carried out on the use of CRISPR-Cas based antimicrobials have been done using near-clonal bacterial populations, a stark contrast to what obtains in real life situations in humans, animals and in the environment, in which bacteria exist in complex communities of diverse genera, species and strain. Even within a particular strain, members differ largely in their plasmids and other mobile genetic elements (MGEs) content, and bear different antibiotics resistance genes [54]. While it is now possible to characterize the genome of bacterial hosts easily, using quantitative Polymerase Chain Reaction (qPCR) and next-generation sequencing technologies, the process of identifying and sorting each antibiotic resistant gene containing pathogen, requires the more cumbersome processes of fluorescence-activated cell sorting of genetically tagged bacteria and MGEs [19]. In addition, it is difficult to predict the response of the mixed microbial community to disturbances as a result of the killing of specific bacterial strains or the disturbances to growth and metabolism that may result from the elimination of certain plasmids as a result of the deployment of the CRISPR-Cas antimicrobial system into the population. Justification of this concern comes from the fact that disturbances in the gut microbiome has been attributed to the development of drug-resistant strains of *Clostridium difficile* [55]. These potentially harmful effects of CRISPR-Cas antimicrobial systems have to be studied in more detail, before their widespread use.

### 6.2 Concerning CRISPR-Cas Delivery Vectors

Currently, the two most viable options of delivery vectors for the CRISPR-Cas antimicrobials include phagemids and conjugative vectors. Each option however possesses its own unique challenges. Bacteriophage-based delivery platforms are powerful vectors for CRISPR-Cas

antimicrobial delivery, but their use is limited by the highly specific/narrow range of infectivity of bacteriophages, making them non-applicable for targeting multiple bacterial strains simultaneously. While efforts to circumvent this challenge such as the use of bioengineering techniques to expand or switch their host ranges have been tested [56,57], there remains a long journey ahead, for this to become common technology. The second option would be the use of conjugative plasmids, however they are also limited by conjugation inefficiencies between bacterial strains [49]; however, their broad host range makes them the more suitable candidates as delivery vectors, with the use of probiotics. A further consideration against the use of conjugative plasmids would be their fitness costs to the bacterial strain, due to the increased genetic burden conferred by the presence of the plasmid. This however can be offset by mutations in both the plasmid and the bacterial host [58–60].

### 6.3 Concerning Safety

Certain components of the CRISPR-Cas system have been reported to have adverse effects on some bacterial species. Naduthodi et al. [61] reported that the Cas9 protein was lethal in *Synechococcus elongatus*, when expressed constitutively. Jiang et al. [62] reported a similar level of toxicity in *Corynebacterium glutamicum*. In both species however, Cas12a was used successfully.

### 6.4 Concerning Resistance

Theoretically, bacterial resistance to CRISPR-Cas based antimicrobials is possible, and can involve either the protospacer being targeted by the system, or the antibiotic resistance gene, especially when the later is under positive selection by the simultaneous presence of an antibiotic [63]. It could also occur through a genetic mutation involving the CRISPR-Cas loci itself, resulting in an inactivation of the *cas* genes encoding the Cas proteins, responsible for target cleavage, as reported by Jiang et al. [64] and Vercoe et al. [65] and are more common than those involving the target sequences or protospacers [49,51]. Furthermore, resistance may be due to the activation of anti-CRISPR (*acr*) genes, which encode proteins that bind to and inactivate the components CRISPR-Cas system [66,67]. More than 20 different families of *acr* genes have been described, affecting both type I and II CRISPR-Cas systems. Those



affecting type I have been described in *Pseudomonas aeruginosa* phages, and while most Acr proteins target a single CRISPR-Cas subtype, a particular Acr has been identified to target both type I-E and type I-F subtypes [68]. Acr proteins targeting the type II systems (the Cas9 family) have also been described [69]. The issue of mutations in target sequences has been resolved by multiplexing, which involves programming the CRISPR-Cas system to target multiple target sequences at the same time, thus reducing the likelihood of effective resistance [70,71], while the problem of selection for *acr* genes can be resolved by using multiple CRISPR-Cas systems of different sub-types simultaneously, taking advantage of the fact that most Acr proteins affect only one CRISPR-Cas subtype [19].

### 6.5 Concerning Regulatory Hurdles

As CRISPR-Cas systems are potent gene editing technologies, their use on human and animal species as well as on environmental settings would require new legislation that breaks the mould of what is currently available [72,73]. It would be important to convince the necessary authorities that deploying such potentially lethal technology is safe and effective. Some important preceding events offer great encouragement that this is indeed possible. Lytic phages have recently been approved as disinfectants for food [74,75], and certain phage-based therapeutics requiring regular optimization have also received approval from the United States Food and Drug Administration (FDA), without the requirement for frequent applications for the same approval each time their constituents need to be altered to stay ahead of resistance [76]. These examples show that it would be possible to navigate the delicate regulatory hurdles ahead for the acceptance of CRISPR-Cas based antimicrobials as conventional therapy in clinical practice.

### 7. CONCLUSION

In summary, while CRISPR-Cas systems have gained widespread acceptance in gene editing techniques, their application as antimicrobial agents remain a path less travelled. Their efficacy has been established by numerous studies and reports however their safe application remains to be established by more to come. In our review, we have highlighted some of their applications and limitations, as well as some techniques that have been proposed to circumvent these limitations, and we believe that

CRISPR-Cas systems will play a pivotal role alongside other established alternatives, if the fight against anti-microbial resistance is to be won.

### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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