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Exploiting Crispr-Cas System to Combat Antibiotic Resistance: A Review

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Abstract: Antimicrobial resistance is a major global health concerns. This is exhibited in bacteria through a combination of acquired and intrinsic processes. An innovative method for managing bacterial strains resistant to antibiotics is the CRISPR-Cas system, a bacterial acquired immune system. This system comprises three regions: directed repeats, spacers and adjacent Cas genes. The three recognized phases in the system's adaptive immunity providing mechanism are adaptation, crRNA biosynthesis and interference. Currently, there are two classes, six types and thirty-three subtypes of the system. PCR, CRSPR-Recognition Tool, CRISPR-Cas Finder, CRISPR Detect and CRISPR identify are among the commonly used system detection tools developed so far. Since the RNA-based spacers bordered by partial repetitions instruct the Cas proteins to precisely target and cleave DNA, encoding matching protospacers, the technique holds great promise for genome editing. This has been harnessed to target bacterial populations carrying antibiotic resistance-coding genes through efficient delivery strategies. While the system can be used to eliminate bacterial resistance genes and/or directly kill bacteria, its existence in the bacterial genome may prevent the acquiring of resistance genes. Despite the progressively increasing development of system, there are possible challenges to be addressed in the future through robust molecular epidemiological studies and tailored delivery systems. Uplifting recent achievements in the microbial world might help enlighten the scientific research community regarding their role in antimicrobial resistance concerns.

Key words: Antibiotic Resistance · Bacteria · Combat · CRISPR-Cas System · Genes

INTRODUCTION

Antibiotic resistance is a major global concern that poses a threat to both the economy and public health. It has been shown to be steadily increasing and new resistance mechanisms appear daily, depleting the availability of existing antibiotics [1, 2]. The main reason of bacterial resistance to antibiotics has a lot of aspects and includes the inherent nature encoded in genes and the attributes gained through evolutionary development [3]. The injudicious overuse of antimicrobials in veterinary and human medicine has made to an increase in the resistance worldwide [2].

Majority of the studies on the effects of the occurrence and spread of antibiotic-resistant bacteria among animals is related to the risk of transfer to humans, thereby posing a potential hazard to public health [4]. There is a lot of information that resistant bacteria such as *Salmonella*, *Campylobacter* and methicillin-resistant *Staphylococcus aureus* can spread between animals and humans. It has been also documented that resistance genes such as the *vanA* gene cluster or genes conveying resistance to higher generations of cephalosporins can spread between animal and human colonizing bacteria [5].

Antibiotic resistance is a worldwide issue, thus vertical efforts under a single-buy business model are not the solutions. It really needs For various sectors, including the pharmaceutical industry, agro-alimentary complexes, patient care and education, governmental and non-governmental organizations and research and development centers, to strategically cooperate, multidisciplinary partnerships are necessary [6]. Different countermeasures have been proposed, including reducing the use of antibiotics and improving surveillance methods or incentives for the pharmaceutical industry to develop new antibiotics. However, the natural challenges of discovering novel antibiotics, in addition to their lack of financial incentive, have caused the discovery of novel

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antibiotics to proceed slowly. Furthermore, the rate at which new antibiotics are developed greatly outpaces the rate at which antibiotic resistance emerges, making the latter insufficient to stem the tide of antibiotic resistance [7, 8].

Hence, through technological dynamics, numerous new approaches have been suggested to reduce antibiotic resistance in pathogens. One of the most studied and proposed approaches that have been developed against antibiotic-resistant bacteria is the clustered regularly inter-spaced short palindromic repeats-CRISPR-associated (CRISPR-Cas) system [1, 9- 11].

CRISPR-Cas system is an acquired immune system that prevents phage infection in prokaryotes by storing memory in bacterial host chromosomes [12, 13]. CRISPR is one of currently a genome editing technique that modifies internal DNA or RNA in a sequence-specific manner and is reprogrammable. CRISPR-associated endonuclease Cas proteins have been used in various ways to precisely modify genes in a process called gene editing. Hence, programmable Cas nucleases such as Cas9, Cas12a and Cas13a in this method has the potential to be fatal or to lessen the resistance of bacteria to antibiotics [14]. Its application in mitigating antimicrobial resistance has been demonstrated in a number of bacteria, including *Staphylococcus aeurus, Escherchia coli* and *Klebsiella pneumoniae* [15, 16]. It is very important to recap such valuable new scientific attainments and apprise the rest of the research community to solve the shooting antimicrobial resistance issue worldwide.

Therefore, this seminar paper is aimed to:

- Point out the mechanism, development and maintenance of antibiotic resistance in bacteria;
- Review the basic biology of the CRISPR-Cas system and
- Review of the utilization of CRISPR-Cas system in combating antibiotic resistance in bacteria.

Antibiotic Resistance in Bacteria

Mechanism of Antibiotic Resistance in Bacteria: The key to resolving this dilemma is comprehending the mechanisms by which bacteria withstand antibiotics [17]. Bacteria can exploit intrinsic, extrinsic, or both mechanistic pathways to develop resistance to antibiotic [18]. When bacteria display resistance, it's because of their innate characteristics. For example, gram-negative bacteria might display glycol-peptide resistance because their cell's outer membrane is impermeable [18]. Drug resistance mechanisms can be classified into several categories. Active efflux pumps are exhibited by most *Enterobacteriaceae* and drug inactivation by producing chemicals such as acetyltransferase, phosphotransferase and adenyltransferase enzymes is characteristic of both gram-positive and gram-negative bacteria. Similarly, modifications of drug-binding targets due to mutations (e.g., *S. pnemoniae* resistance to penicillin), changes in cell permeability resulting in reduced intracellular drug accumulation and changes in metabolic pathways are mostly manifested [3, 19].

On the other hand, acquired resistance happens when a previously susceptible bacteria develops resistance as a result of a mutation or the horizontal gene transfer of new genetic material from external sources. Resistance gene-carrying plasmids and conjugative transposons play a significant role in this transfer in both gram-positive and negative bacteria [19]. In addition, such resistance can also be developed via adaptation to one or more antibiotics induced by specific environmental signals such as stress, growth state, pH, ion concentrations and sub-inhibitory levels of antibiotics [18].

Evolution and Maintenance of Antibiotic Resistance in Bacteria: The ability of bacteria to evolve through horizontal gene transfer mechanisms, such plasmid insertion, has facilitated their capacity to adapt to novel settings [15, 20]. One of the main theories for the occurrence of resistance to multiple drugs (MDR) in harmful bacteria is this type of recombination. Genes encoding enzymes that break down or release medicines are usually responsible for high levels of drug resistance. These genes are usually gained from other microorganisms during gene exchange [21].

Additionally, it has been demonstrated that low-to-moderate resistance can develop from scratch, occasionally after only a short period of medication exposure [22]. The lowest inhibitory level can be increased by tens of times or more by one nucleotide polymorphism that modifies the antibiotic's attachment site and by a few thousand times if multiple such alterations are combined [23].

Detection and Targeting of Antibiotic Resistance Genes in Bacteria: Besides PCR [15, 24], numerous CRISPR-Cas technologies have been designed to identify genetic materials and biomarkers in bacteria thereby helping in the detection of antibiotic resistance genes in bacteria. They can be employed to precisely detect antibiotic resistance and virulence genes in pathogenic bacteria, as

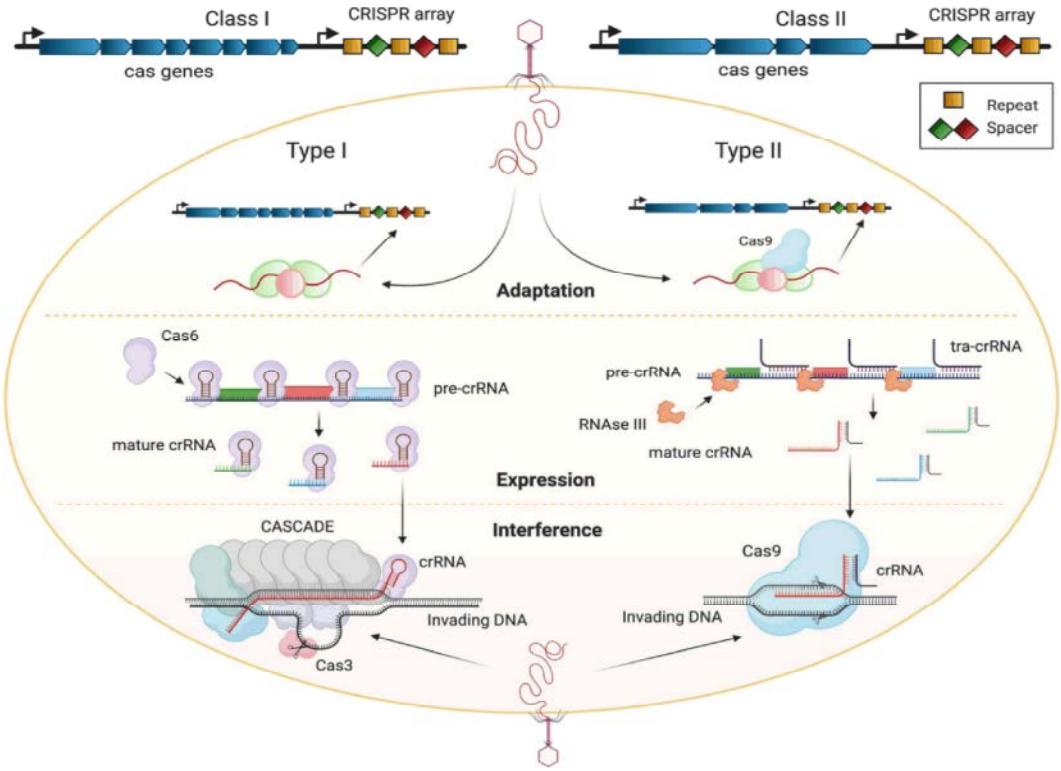

Fig. 1: The core elements and immune mechanisms of CRISPR-Cas system [28]

well as genotypes and single nucleotide polymorphisms in pathogenic bacteria. They can also be used to determine bacterial infections [1]. This method was developed based on optical DNA mapping combined with Cas9-assisted identification of resistance genes, which was previously used to characterize plasmids during an extended spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* outbreak [25].

Unlike conventional nucleic acid detection technologies, CRISPR-Cas system enables on-site detection because it uses lateral flow assays, which are straightforward, portable and independent of specialized equipment or a particular setting. It takes only a few hours to see the results thanks to a time-saving method that integrates CRISPR-based reaction systems with fluorophores, quenchants, nanoparticles and turbidity changes. Moreover, compared to quantitative polymerase chain reaction (qPCR), which is frequently regarded as the gold standard, this method has higher sensitivity and specificity [25]. Furthermore, CRISPR-Cas technology permits the simultaneous detection of several target molecules; as a result, it is currently recognised as a state-of-the-art approach for next-generation diagnostics that concurrently satisfy numerous test criteria [1].

Description of Crispr-cas System

Biology and classification of CRISPR-Cas System

Biology of CRISPR-Cas System: Clustered regularly interspaced short palindromic repeats-Cas (CRISPR-Cas) systems have been identified as bacterial adaptive immune system [26]. The system is found in approximately 40% and 85% of bacterial and archaeal genomes, respectively [13]. The genetic loci of CRISPR-Cas systems (Figure 1) contain the CRISPR array, which comprises short repeated sequences (repeats of 23-55 base pairs) and similarly sized flanking sequences (spacers of 26-72 base pairs). The spacers of CRISPR arrays are protospacers and are acquired from either invading phages or plasmids. The repeat sequences defined each CRISPR locus, with the most prevalent repeat inside the locus being referred to as a typical repeat. Within the same strain, each spacer has a distinct sequence and different strains of the same microbial species have different numbers of repeats in their CRISPR. Cas proteins are essential components of CRISPR systems that determine their activity; they are encoded upstream of the CRISPR array [13, 27].

Table 1: Classes of CRISPR-Cas system along with functional steps and effector complexes

		Adaptation	Expression	Interference	
Classes	Types and subtypes	Spacer integration	Pre-crRNA processing	Effector complex	Target cleavage
Class 1	Type I (A, B, C, D, E, F, G)	Cas1, Cas2, Cas4	Cas6	Cas7, Cas5, SS, Cas8/LS	Cas3", Cas3'
	Type III (A, B, C, D, E, F)	Cas1, Cas2, RT	Cas6	Cas7, Cas5, SS, Cas10/LS	Cas10/LS
	Type IV (A, B, C)	Cas1, Cas2	Cas6	Cas7, Cas5, SS, Csf1/LS	?
Class 2	Type II (A, B, C, D)	Cas1, Cas2, Cas4	RNase III	Cas9	Cas9
	Type V (A, B, C, D, E, F, G, H, I, K)	Cas1, Cas2, Cas4	Cas12	Cas12	Cas12
	Type VI (A, B, C, D)	Cas1, Cas2	Cas13	Cas13	Cas13

Key: RT, reverse transcriptase; SS, small subunit; LS, large subunit. = unknown
Source: (12)

Overall, the CRISPR-Cas system mediates immunity against invading genetic elements (phages, transposons and plasmids) via a three-step process: adaptation, expression and interference. Several nucleotides long protospacer-adjacent motifs of the CRISPR/Cas system recognise short segments of DNA similar to the virus or plasmid sequences, which are incorporated into the CRISPR locus with the help of Cas proteins during the adaptation step. The long primary transcript of the CRISPR locus (pre-crRNA) is produced and converted into small crRNAs during the expression stage. Third-stage foreign DNA or RNA is guided by crRNAs linked to Cas protein complexes to be targeted and cleaved within the protospacer sequence [27]. Upon binding of crRNA to its cognate DNA target, the Cas protein generates a double-stranded DNA break in the target [28].

Classification of CRISPR-Cas System: According to the current classification in the study by Makarova *et al.*, there are two classes of CRISPR-Cas system, which include six types (I-VI) and 33 subtypes. Sequence similarity, phylogenetic analysis, neighbourhood analysis and comparison, unique component features and experimental data including unique physiology, biochemistry and molecular mechanism features are used to describe six different types of CRISPR-Cas systems [13].

Class I comprises types I, III and IV as well as sixteen subtypes that have several Cas proteins acting as effector modules to mediate pre-crRNA processing and interference and produce crRNA-binding complexes. Types II, V and VI are included in class 2, as are 17 subtypes that have a single, large, multidomain crRNA-binding protein (Cas9 in type II, Cas12 in type V and Cas13 in type III). These subtypes are involved in all the activities necessary for pre-crRNA processing (in some variants) and interference (in all variants). The classes along with their functional steps and effector complexes are summarized in (Table 1) [12, 13].

Detection and Identification of CRISPR-Cas System in Bacterial Genomes: The prevalence of CRISPR/Cas can be determined by PCR, using primers for the respective CRISPR-Cas loci [15]. A growing number of scientists are concentrating on creating computational methods for the detection of CRISPR-Cas systems due to the quick growth of bioinformatics and the growing necessity of the CRISPR-Cas system. The following CRISPR sequence identification tools, which mainly rely on repeating structures in arrays, have been developed to date: CRSPR recognition tool, CRISPR-Cas Finder, CRISPR Detect, CRISPR identify, Metagenomic CRISPR reference-aided Search Tool (Meta-CRAST), CRISPR/Cas Typer and CRISPR leader. These identification tools have been used to explore the diversity and classification of the CRISPR-Cas system [14].

Gene editing mechanism of the CRISPR-Cas System: Although both classes of CRISPR systems have significant potential for genome editing, class 2 systems are more prudently applicable because their effector complexes are simpler [12]. In order to "knockout" a specific gene, the genome editing process entails completely eliminating gene expression and creating double strand breaks in DNA. On the other hand, gene silencing involves "knockdown" of the target gene utilising catalytically dead Cas9 (dCas9), which lacks endonuclease activity [29, 30]. Moreover, engineered CRISPR-Cas systems have been reported to effectively kill bacteria and even reverse resistant bacteria to an antibiotic-susceptible state [1].

Cas proteins are generally directed to selectively target and cleave DNA encoding for matching protospacers by short guide RNA-based spacers bordered by incomplete repeats. Because the CRISPR array contains information that may be used to specifically target and cleave any DNA in vivo, the CRISPR-Cas system has been used to target the bacterial population that carries particular genes that encode antibiotic resistance [9, 16, 31].

Table 2: Delivery strategies of CRISPR-Cas system with respective targets of organisms

№	Strategy	CRISPR-Cas	Organism	Target gene	Result	Reference
1	Phage-based	CRISPR-Cas9	*S. aeurus*	bla_{NDM-1} and bla_{SHV-18}	Death	[32]
		CRISPR-Cas9	*S. aeurus*	*mecA*	Sensitivity	[16]
		CRISPR-Cas9	*E. coli*	*Intimin*	Death	[32]
		CRISPR-Cas9	*S. aeurus*	*agrA*	Death	[47]
		λcas-CRISPR	*E. coli*	bla_{NDM-1} and $bla_{CTX-M-15}$	Inhibit transfer	[31]
2	Conjugative-based	CRISPR-Cas9	*S. enterica*	*katG, yghJ, aegA* and *gltJ*	Death	[43]
		Type I-E	*E. coli*	*fucP*	Death	[48]
		CRISPR-Cas9	*E. faecalis*	*tetM* and *ermB*	Inhibit transfer	[49]
3	Nanoparticle	CRISPR-Cas9	*S. aeurus*	*mecA*	Death	[33]

CRISPR-Cas Delivery Strategies: Recent studies have shown that intentional or accidental targeting of the bacterial genome sequence by the CRISPR-Cas system is cytotoxic and can lead to cell death due to the introduction of irreversible chromosomal lesions [32]. The CRISPR-Cas system must therefore be isolated, optimised and delivery vectors and carriers must be developed because the CRISPR-Cas system is highly conserved among bacteria and archaea. The development of RNA-guided nucleases that can target new strains, such as pathogens resistant to drugs and important endogenous microbiota members, depends on such delivery mechanisms [14, 32]. This has been mostly achieved using polymer-derivatized CRISPR nanocomplexes [33], bacteria carrying plasmids transmissible by conjugation [32] and/or bacteriophages (Table 2) [16, 32].

Phage-based Delivery: Several studies have described and used phage-based CRISPR-driven techniques for the prevention of bacterial drug resistance [31,32,34,35]. In this approach, bacteriophages are designed to carry and deliver CRISPR-Cas to combat multidrug-resistant bacteria. Packaging renders the delivery of encapsulated DNA highly efficient because it uses a proficient phage injection mechanism. Such systems are currently being developed by biotechnology companies such as Locus Biosciences (Morrisville, USA) and Eligo Bioscience (Paris, France) [36, 37]. They are made to incorporate CRISPR-targeted bacterial genes into the temperate phage genomes, enabling the phage to inject its own genome into bacteria to finish the bacterial invasion [38].

A study conducted on *P. aeruginosa* showed that single-nucleotide mutations in the phage tail fibrin lead to host-specific changes [39]. Globus *et al*. [40] also used temperate type-7 (T7)-derived defective phages to enhance DNA transduction to various bacteria by mutating the tail fiber gene in the phage plasmid, allowing the phage to lose its ability to expand its host range. Park et al. extended öSaBov host ranges by supplementing the gene encoding the tail fiber protein from ö11 (orf50) [41]. These results suggested that phage host specificity could be regulated by modifying the phage tail protein.

Conjugative-based Delivery: One intriguing delivery method for CRISPR nucleases is the conjugative plasmid delivery mechanism. Conjugative plasmids are resistant to restriction-modification systems, have a wide host range, don't require cellular receptors and are simple to construct with huge coding capacities [14]. Due to increased cell-to-cell contact, conjugative plasmids that encode and encourage the creation of biofilms may accelerate the rates of conjugative plasmid transfer. This could make them suited for the delivery of CRISPR nucleases to alter the makeup of microbial communities that already exist as biofilms [42].

Hamilton *et al*. [43] developed a conjugative system in which the plasmid encoded both CRISPR nuclease and conjugative machinery. Then CRISPR nuclease, under conditions that enhance cell-to-cell contact, had a higher frequency of conjugative transfer from *E. coli* to *Salmonella enterica*. Thus, it was concluded that single or multiplexed sgRNAs targeting additional non-essential genes, such as *katG* (catalase reductase), *yghJ* (putative lipoprotein), *aegA* (putative oxidoreductase) and *gltJ* (glutamate/aspartate transporter), result in a higher killing efficacy of *S. enterica* compared to the essential genes of the same pathogen [14]. In addition, Citorik *et al*. [32] demonstrated that treatment of enterohemorrhagic *E. coli* (EHEC) with phagemideae targeting *intimin*, which is a chromosomally encoded virulence factor of *E. coli* O157:H7 that is necessary for intestinal pathology and colonization resulted in a 20-fold reduction in viable cell counts.

Nanoparticles-based Delivery: The application of nanotechnology for the synthesis and delivery of new antibiotics is an important approach [36, 44]. Delivering CRISPR-Cas9 has become feasible and helpful thanks to the development of nanoparticle technologies. It has been

demonstrated that adjustable sizes of nanoparticle-based vectors can be used to package CRISPR-Cas systems and preserve the original nanostructures during gene transfer. They also give nucleic acid molecules an anti-degradation barrier and are safer than viral vectors due to their biocompatibility, surface functionalization, reduced immunogenicity and other advantages [45]. Lipid nanoparticles, polymeric nanoparticles and gold nanoparticles are among the frequently used nanoparticles so far [14, 33, 46].

Role of CRISPR-Cas System in Combating Antibiotic Resistance

CRISPR-Cas System and Natural Resistance of Bacteria to Antibiotics: Studies have shown a significant reverse relationship between the CRISPR-Cas system and antibiotic resistance in certain bacterial species. Other studies have shown a positive relationship between the CRISPR-Cas system and antibiotic resistance [14]. Several bacterial species and/or strains, as well as different CRISPR-Cas effectors, were used in the experiments. CRISPR loci are extensively accumulated in strains of Enterococcus faecalis that are vulnerable to erythromycin, gentamycin, teicoplanin and tetracycline. Additionally, it has been observed that in strains that were positive for ant (6), vanA, tetM, ermB, aac6'-aph (2") and tetM, fewer CRISPR loci were found, which is consistent with drug susceptibility. These findings show a negative relationship between antibiotic resistance, CRISPR-cas loci and the presence of antibiotic-resistant genes [50].

Similarly, it has been reported that, in *Shigella*, *cas1* and *cas2* mutations contribute to different levels of resistance. Point mutations at sites 3176455, 3176590 and 3176465 in *cas1* (a); sites 3176989, 3176992 and 3176995 in *cas1* (b); and sites 3176156 and 3176236 in *cas2* may affect the resistance of bacteria, cause emergence of multidrug resistance and increase the types of drug resistance [51]. In other bacteria, such as *Mycobacterium tuberculosis* complex, *Cas1* has been found to increase the sensitivity of bacteria to multiple anti-tuberculosis drugs by reducing their persistence during drug treatment [52]. Furthermore, an *in vitro* resistance development assay in *Acinetobacter baumannii* revealed that the complete CRISPR-Cas system could inhibit the development of bacterial resistance. This is because the *csy1* gene of the system has been found to be the main role player [53].

In contrast, a study conducted on multidrug-resistant (MDR) isolates of *Salmonella enterica, Serovar enteritidis* found a positive relationship between CRISPR-Cas system and antibiotic resistance [54]. There have been other previous studies supporting this finding [55], in *Campylobacter jejuni cas9* was found to be involved, as was *Francisella novicida* [56].

The aforementioned controversies may be related to the Cas genes involved, the species of bacteria harboring species-specific spacers and evolutionary modifications among other related factors [14].

Interference with Transfer of Antibiotic Resistance Genes: Pathogenic bacterial strains have emerged largely because of the transfer of virulence and antimicrobial resistance genes among bacteria via horizontal gene transfer. The CRISPR loci of bacteria encode a sequence-specific defense mechanism against bacteriophages and constitute a programmable barrier to horizontal gene transfer [9, 57].

Maraffifini and Sontheimer [59] demonstrated that *S. epidermidis* might have plasmid conjugation limited by the CRISPR-Cas system, suggesting a broader and more critical role of the CRISPR-Cas system in the prevention of horizontal gene transfer. Using methicillin-sensitive *S. aureus* as the research target, Bikard *et al*. [16] inoculated a phagemid to target a plasmid containing the tetracycline resistance gene and transferred the plasmid into treated cells; however, no tetracycline resistance was observed. Subsequently, Yosef *et al*. [31] delivered the CRISPR-Cas9 system into *E. coli* using λ phage as a vector and successfully destroyed plasmids carrying the *bla*NDM-1 and *bla*CTX-M-15 genes. Moreover, compared to the negative control lamda *cas* (*λcas*), which lacks CRISPR, the plasmid transformation efficiency of these lysogens was greatly reduced, effectively preventing the transfer of antibiotic resistance elements.

Furthermore, a study on clinical *Klebsiella pneumonae* indicated that strains containing subtype I-E CRISPR-Cas systems had decreased numbers of plasmids, prophage regions and acquired antibiotic resistance genes in their published genomes. Further investigation of the same pathogen revealed that the subtype I-E CRISPR-Cas system in *K. pneumoniae* potentially interferes with the acquisition of phages and plasmids harboring antibiotic resistance determinants, thus maintaining these isolates susceptible to antibiotics (amikacin, gentamicin and levofloxacin) [55].

Similarly, two years later another study showed that ESBL-producing and carbapenem-resistant *K. pneumoniae* were more likely to develop multidrug resistance and showed an inverse correlation between drug resistance and the CRISPR/Cas system.

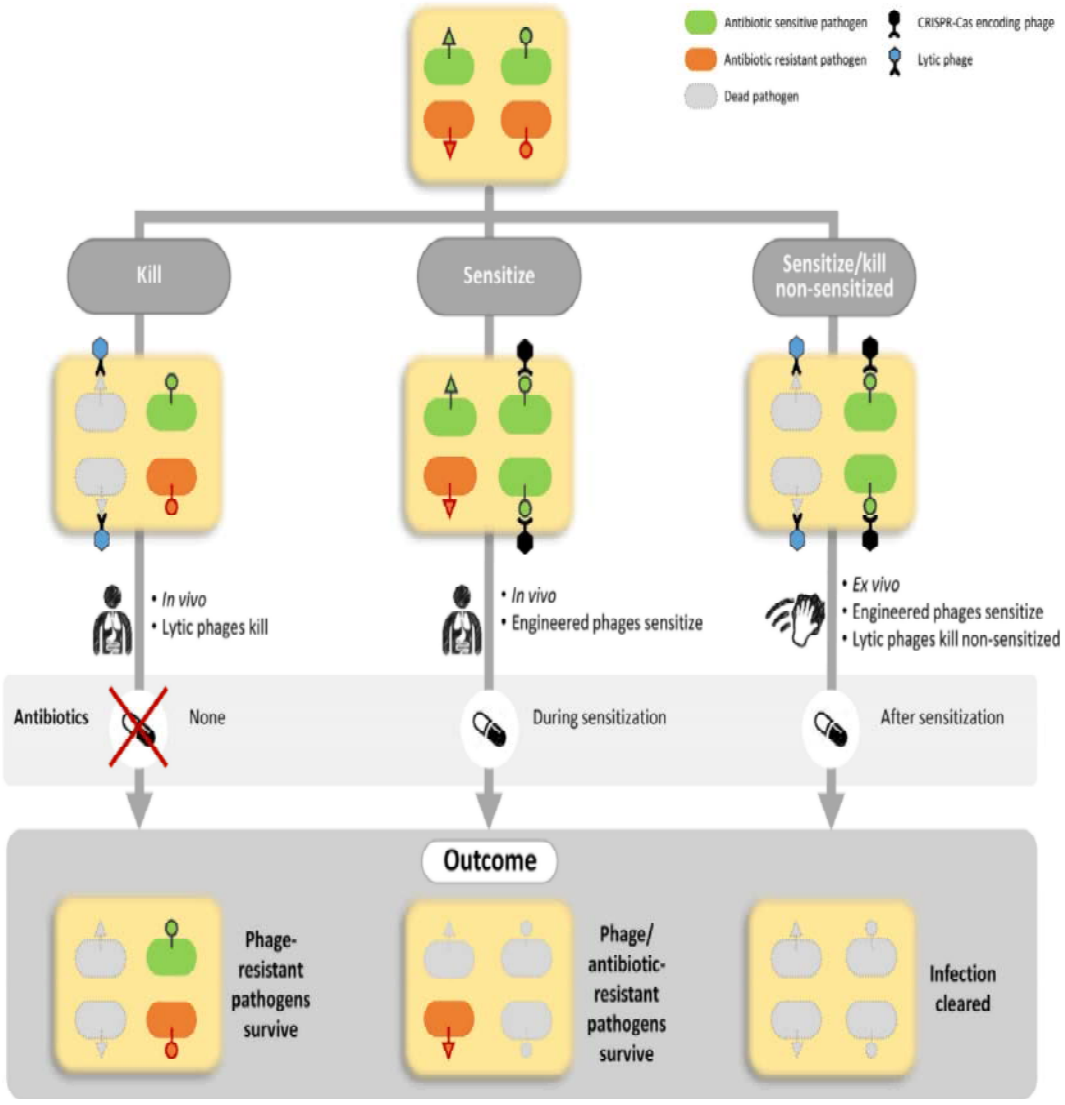

Fig. 2: Selective killing of antibiotic resistant over sensitive bacteria [10]

These studies suggest that the absence of CRISPR/Cas modules allows these bacteria to acquire external drug resistance genes [15]. Similar roles identified on *Streptococcus pyogenes*, *Pseudomonas aeruginosa* and *Escherchia coli* [9, 58].

Therefore, CRISPR-Cas-based "vaccine" design to prevent drug resistance genes from entering antibiotic-sensitive bacteria is worthy of in-depth study by scientists to prevent the spread of antibiotic resistance via horizontal gene transfer. Similarly, CRISPR-Cas system-mediated targeted elimination of antibiotic-resistant genes may be a potential tool for the clinical control of drug resistance gene transmission and drug-resistant pathogens [1].

Removal of Antibiotic Resistance Genes: The CRISPR-Cas system acts as a nuclease that can be guided to cleave any target DNA, allowing sophisticated yet feasible manipulation of pathogens [59]. Goren *et al*. [10] reviewed pioneering studies that used the CRISPR-Cas system to specifically edit bacterial populations and eliminate their resistance genes. Therefore, artificial selection pressure for antibiotic-sensitive pathogens was established using a combination of these two strategies. Hence, it has been suggested the intelligent design of this system, along with efficient delivery tools for pathogens, may significantly reduce the threat of antibiotic-resistant pathogens [14].

Experiments targeting resistance genes using the CRISPR-Cas system were reported soon after the system was first characterized [60]. Citorik *et al*. [32] used *E. coli* infecting phage (M13-based) phagemids to target antibiotic resistance plasmids in pathogenic *E. coli*, rendering the targeted bacteria vulnerable to antibiotic action. Bikard *et al*. [16] used *S. aureus* as a model pathogen and its ÖNM1 phage for the phagemid encapsidation. Phagemids encode spacers that target genes that confer antibiotic resistance to *S. aureus*. It has been shown that delivery of spacers by phagemids efficiently cured plasmids encoding these resistance genes in more than 99% of bacteria. This resulted in significant sensitization to kanamycin. This has also been demonstrated for methicillin [33].

Yosef *et al*. [31] introduced another novel technology with selective pressure for antibiotic sensitivity in antibiotic-resistant bacteria (*E. coli*). In this study, temperate phages were used to deliver a functional CRISPR-Cas system to the genomes of antibiotic-resistant bacteria. Here, we show that the delivered CRISPR-Cas system destroys both antibiotic-resistance-conferring plasmids and genetically modified lytic phages. This linkage between antibiotic sensitization and protection from lytic phages was a key feature of the strategy and allowed the programming of lytic phages to kill only antibiotic-resistant bacteria while protecting antibiotic-sensitized bacteria (Figure 2) [10].

Direct killing of the bacteria: Contrary to conventional antibiotics, which frequently lack specificity, CRISPR-Cas targets specific antibiotic resistance genes and removes harmful bacteria from complex bacterial communities without impacting other bacterial species [35]. The CRISPR-Cas system's capability to target chromosomes determines how effectively it can kill harmful germs directly [14] and plasmids [32].

CRISPR-Cas3- and Cas9-systems provide a means to combat such threats by selectively killing antibiotic-resistant bacteria [37]. CRISPR-Cas3 and CRISPR-Cas9 genome-editing constructs, designed to target antibiotic resistance genes, were delivered into bacteria by packaging them into phages [31, 35, 47]. Both constructs were applied to achieve antibiotic-resistant gene-specific bacterial killing, for example in *S. enterica* [31, 43].

In *in vivo* experiment, antibacterial drones with either a CRISPR-Cas9 bactericidal or CRISPR-dCas9 virulence-blocking module were constructed. This demonstrated that both antibacterial drones blocked the development of a murine subcutaneous *S. aureus* abscess. This bactericidal module rescued mice that had been intraperitoneally administered a lethal dose of *S. aureus* intraperitoneally [47]. Similarly, CRISPR/Cas9 targeting of the *E. faecalis* erythromycin resistance gene *ermB* significantly reduced the overall presence of erythromycin-resistant *E. faecalis* in the guts of mice [49]. Studies have also shown that CRISPR-Cas9 performs a particular role in eliminating target genes on bacterial chromosomes, which has been confirmed in *S. aureus* [14], *Salmonella* [48] and *Streptococcus pneumoniae* [57].

More importantly, the development of a series of CRISPR-Cas13a-based antibacterial nucleocapsids, termed CapsidCas13a(s), has recently been reported. This series was confirmed to be capable of sequence-specific killing of carbapenem-resistant *Escherichia coli* and methicillin-resistant *Staphylococcus aureus* by recognizing corresponding antimicrobial resistance genes. Capsid Cas13a constructs were generated by packaging programmed CRISPR-Cas13a into a bacteriophage capsid to target antimicrobial resistance genes [34]. Capsid Cas 13a(s) exhibit strong bactericidal activity upon recognizing target genes, regardless of their location [14].

Challenges and Future Perspectives

Challenges: Through technological development, not everything might be straightforward until its application becomes extensive. Considerable challenges have been raised at different points of development and have been addressed in a number of studies.

Multifaceted Microbial Populations: Although it is obvious that CRISPR-Cas has a great potential for sequence-specific killing or re-sensitization of bacteria carrying antibiotic resistance genes, the effectiveness of using CRISPR-Cas to delete these genes has only been studied in close-knit bacterial populations. In real-world settings, where bacteria are frequently part of microbial communities, using such a method [61], is far more challenging. Millions of different species make up the billions of cells per gram of matrix present in the natural microbial communities found in the human, animal and environmental microbiomes. Even within a single species or strain, clonal lineages may contain several plasmids and other mobile genetic components that bear a variety of resistance genes [62].

Resistance progression against CRISPR-Cas: The development of resistance is another problem that can obstruct the successful implementation of CRISPR-Cas. In CRISPR-phage interactions, this is known to readily occur

through the acquisition of point mutations in the sequence targeted by CRISPR-Cas [62]. In principle, this may also happen in genes that are resistant to antibiotics and might be targeted for deletion, especially if these genes are being positively selected (e.g. when antibiotics to which the antibiotic resistance gene confers resistance are used). Alternatively, resistance could evolve by inactivation of CRISPR-Cas loci through mutations or deletions in *Cas* genes which are essential for target cleavage or by deleting targeting spacers [57, 62].

Apart from the evolution of resistance through mutations, resistance can also evolve through the selection of anti-CRISPR genes, which encode small proteins that bind to and inactivate critical components of the CRISPR-Cas immune system. At present, over 20 unique families of anti-CRISPR genes targeting both type I and II CRISPR-Cas systems have been identified [63].

CRISPR-Cas System and Future Perspectives: Despite the various challenges to the wide application of the CRISPR-Cas system as a tool to tackle antibiotic resistance, advancements in science and technology have assisted in the development of potential solutions to circumvent some of these challenges. The most pressing issue facing the use of CRISPR-Cas-mediated removal of antibiotic resistance is finding an appropriate delivery vector [32] tailored for specific purposes. The improvement of these tailored delivery systems would be a step towards tackling the challenge of the complexity of microbial communities if a suitable broad host range vector can be identified or engineered [62].

The ecological consequences of using CRISPR-Cas antimicrobials in a community context must be carefully studied by monitoring the effects of the removal of antibiotic resistance genes on the frequency of other bacterial species and their associated plasmids. In long-term applications, the evolution of resistance is inevitable. However, resistance through the mutation of target sequences can potentially be avoided by multiplexing, which involves CRISPR-Cas targeting multiple sequences simultaneously to reduce the likelihood of resistance [64]. Moreover, the selection of anti-CRISPR genes may be mitigated by using multiple CRISPR-Cas variants simultaneously, which would require different anti-CRISPRs to escape targeting or engineering anti-CRISPR-insensitive CRISPR-Cas variants. An approach using nucleases as alternatives to Cas9, such as Cas12a, may also circumvent any issues with the toxicity and efficacy of the system in various bacterial hosts [62].

In addition to technical study approaches, community engagement is an important step, both to receive advice on best practices and to gauge public and stakeholder support for the use of such technologies [62, 65].

CONCLUSION AND RECOMMENDATIONS

The challenge of antibiotic resistance is alarmingly prevalent worldwide and its mitigation requires multisectoral efforts. Expressed by different mechanisms, resistance is encoded by certain bacterial genes with the possibility of evolution and persistence. Currently, the genome editing capability of the CRISPR-Cas system and bacterial adaptive immunity have paved the way to combat antibiotic resistance. This system can be exploited to quantitatively and selectively eliminate individual bacterial strains in a sequence-specific manner, creating opportunities for intervention in MDR infections. This has led to the development of efficient delivery strategies. Nevertheless, as a young approach, it has been facing immense challenges, such as the complexity of the microbial community and the evolution of resistance against the system. Despite many challenges, the application of this system is advancing progressively to alleviate the challenges of antibiotic-resistant bacteria. Accordingly, the following points were proposed:

- The diversity of different Cas genes in different species and/or strains of bacteria may play different roles in antibiotic resistance; therefore, detailed studies should be conducted.
- The ecological consequences of using the CRISPR-Cas system should be assessed in detail to minimize negative effects.
- Alternative and tailored CRISPR-Cas delivery strategies should be designed to address the complex microbiome.
- Multiplexing is an important strategy for addressing CRISPR-Cas-resistant variants; therefore, additional investigations are necessary.

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