ACS Infectious Diseases

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Review

CRISPR-Cas-Based Antimicrobials: Design, Challenges, and Bacterial Mechanisms of Resistance

Published as part of the ACS Infectious Diseases virtual special issue "Drug Resistance in Infectious Diseases and Beyond".

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Cite This: *ACS Infect. Dis.* 2023, 9, 1283−1302

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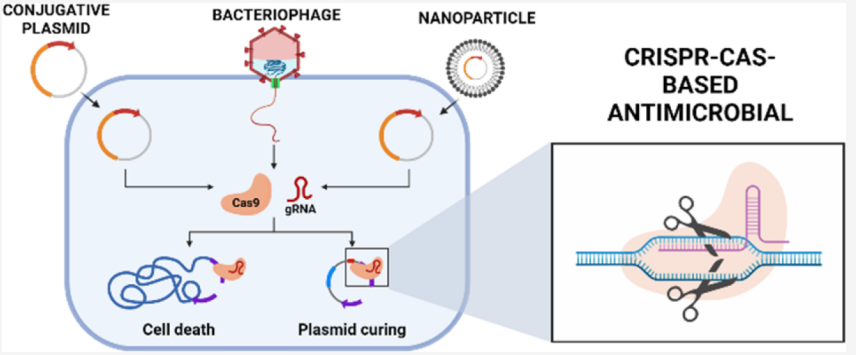

ABSTRACT: The emergence of antibiotic-resistant bacterial strains is a source of public health concern across the globe. As the discovery of new conventional antibiotics has stalled significantly over the past decade, there is an urgency to develop novel approaches to address drug resistance in infectious diseases. The use of a CRISPR-Cas-based system for the precise elimination of targeted bacterial populations holds promise as an innovative approach for new antimicrobial agent design. The CRISPR-Cas targeting system is celebrated for its high versatility and specificity, offering an excellent opportunity to fight antibiotic resistance in pathogens by selectively inactivating genes involved in antibiotic resistance, biofilm formation, pathogenicity, virulence, or bacterial viability. The CRISPR-Cas strategy can enact antimicrobial effects by two approaches: inactivation of chromosomal genes or curing of plasmids encoding antibiotic resistance. In this Review, we provide an overview of the main CRISPR-Cas systems utilized for the creation of these antimicrobials, as well as highlighting promising studies in the field. We also offer a detailed discussion about the most commonly used mechanisms for CRISPR-Cas delivery: bacteriophages, nanoparticles, and conjugative plasmids. Lastly, we address possible mechanisms of interference that should be considered during the intelligent design of these novel approaches.

KEYWORDS: *CRISPR-Cas, antimicrobial design, antibiotic resistance*

INTRODUCTION

The spread of antibiotic-resistant bacteria is a source of major global concern as it is a rising cause of fatal therapeutic failures, and this is anticipated to continue increasing in the future.[1] On the other hand, the discovery of new antibiotics has stalled significantly over the past decade, and the development of novel approaches to address the spread of antimicrobial resistance genes demands substantial efforts.[2,3] This dichotomy emphasizes the need to develop innovative antimicrobial strategies as a viable alternative to existing antibiotics.[4] One major disadvantage of conventional antibiotics is the lack of species specificity, as they can induce metabolic and structural damage into large bacterial communities of both beneficial and pathogenic strains.[5] Additionally, the literature has linked antibiotic selection pressure to the dramatic enrichment of drug-resistant strains in all areas of the biosphere.[6−8]

Using a CRISPR (Clustered Regularly Interspersed Short Palindromic Repeats)-Cas system for the precise elimination of targeted bacterial populations holds promise as an innovative approach for new antimicrobial agent design.[9] The specificity

Received: December 31, 2022
Published: June 22, 2023

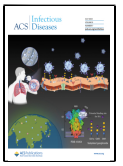

Table 1. Current CRISPR-Cas-Based Antimicrobials in Development[a]

CRISPR-Cas system (type)	Pathogen	Delivery system	Target location	Gene target	Refs
		In Vivo Study			
CRISPR-Cas9 (Type II)	*E. coli*	Cri-nanocomplex (carbon quantum dots)	Chromosome (*pap* gene cluster)	*papG*	53
	E. coli	Conjugative plasmid (TP114 plasmid)	Chromosome (AMR cassette inserted in *glmS* terminator)	*cat*	54, 55
	E. faecalis	Conjugative plasmid (PRP pPD1)	Plasmid (pAM771, pCF10)	*ermB* *tetM*	56
	E. faecalis	Conjugative plasmid (PRP pPD1)	Plasmid (pAM714)	*repB*	57, 58
	S. aureus	Bacteriophage (temperate phage)	Chromosome (thermonuclease encoding region)	*nuc*	59, 60
	S. aureus	MGEs (staphylococcal pathogenicity islands [SaPIs])	Chromosome (*agr* and listeriolysin encoding regions)	*agrA* *hly*	61
	S. aureus	Bacteriophage (phiNM1 phage)	Chromosome (kanamycin resistance encoding regions)	*Aph-3* *mecA*	62
CRISPR-Cas3 (Type I)	*C. difficile*	Bacteriophage (temperate phage)	Chromosome (CR11 array)	ND	63
		In Vitro Study			
CRISPRi (derived from CRISPR-Cas9)	*E. coli*	Conjugative plasmid (recombinant plasmids)	AcrAB-TolC-associated mRNAs	*acrA, acrB* *tolC*	44
	M. abscessus	Conjugative plasmid (pLJR962)	Peptidoglycan biosynthesis-associated mRNAs	*pbpB* *cwIM*	64
CRISPR-Cas9 (Type II)	*E. coli*	Conjugative plasmid (pSC101)	Plasmid (pNDM-5)	*blaNDM-5*	65
	E. coli	Conjugative plasmid (suicide plasmid)	Plasmid (multiple AMR-bearing plasmids)	*mcr-1* *blaKPC-2* *blaNDM-5*	66
	E. coli	Conjugative plasmid (transposon-associated suicide plasmid)	Chromosome and plasmid (colistin resistance encoding regions)	*mcr-1*	67
	Enterobacteriaceae spp.	Conjugative plasmid (pSB1C3)	Plasmid (pSB1A2)	*blaTEM-1*	68
	E. coli	MGEs (pro-active genetic system)	Plasmid (*bla* harboring pET)	*bla*	69
	E. faecium	Conjugative plasmid (pVDM1001)	Chromosome (*lac* operon encoding region)	*lacL*	70
	S. aureus	Nonconjugative plasmid (Apa I-cut pLI50)	Chromosome (WTA biosynthesis encoding regions)	*tarO* *tarH* *tarG*	71
	S. aureus	Cri-nanocomplex (polymer-derivatized SpCAS9)	Chromosome (PBP2a encoding regions)	*mecA*	72
CRISPR-Cas3 (Type I)	*E. coli*	Bacteriophage (λ phage, T7 phage)	Plasmid (pNDM, pCTX)	*ndm-1* *ctx-M-15*	73
CRISPR-Cas13a (Type IV)	*E. coli*	Phagemids (Cas13a encapsulated with bacteriophage capsid)	Chromosome and plasmid (multiple AMR-encoding regions)	*blaIMP-1* *blaOXA-48* *blaVIM-2* *blaNDM-1* *blaKPC-2* *mcr-1 mcr-2*	47

[a]Abbreviations: AMR, antimicrobial resistance; Cri-nanocomplex, nanosized CRISPR complex; MGE, mobile genetic element; ND, nondisclosed; PBP2a, penicillin-binding protein 2a; PRP, pheromone-responsive plasmid; WTA, wall teichoic acid.

of the CRISPR-Cas approach relies upon the interaction between the Cas protein and a guide RNA (gRNA) sequence designed for targeting unique DNA sequences within the pathogenic target strain.[10] The Cas nuclease/gRNA complex can then induce double-strand breaks (DSBs) in the selected sequence, which can lead to cell death or the loss of the targeted plasmid (plasmid curing).[11] Under this premise, it is crucial to develop appropriate strategies to deliver the CRISPR-Cas complex into all bacteria of the target population and to foresee possible mechanisms of interference or resistance.

CRISPR-Cas ANTIMICROBIALS: ORIGIN AND DESIGN

CRISPR-Cas: The Adaptive Bacterial Immune System.

Bacteria have shown several strategies to prevent their damage or death by antibiotics, including the use of efflux pumps to deplete the antibiotic from the cell, the use of specific enzymes

https://doi.org/10.1021/acsinfecdis.2c00649
ACS Infect. Dis. 2023, 9, 1283−1302

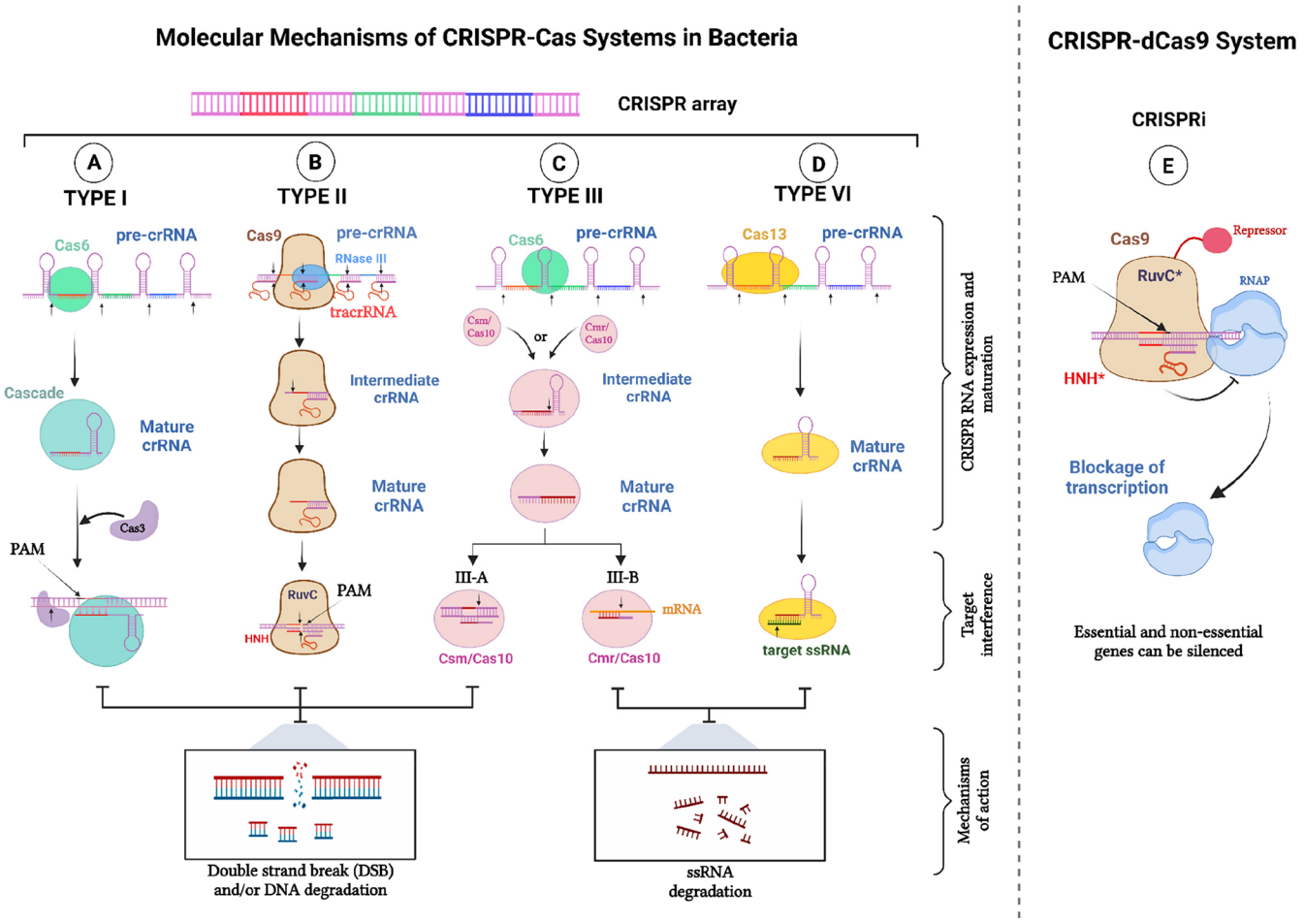

Figure 1. CRISPR-Cas systems employed for developing next-generation antimicrobials. Left panel shows the molecular mechanisms of CRISPR-Cas systems present in bacteria that have been used to develop CRISPR-based antimicrobials (A, B, C, and D). Right panel shows a strategy based on the type II system with a mutant Cas9 nuclease (dCas9) also used to develop CRISPR-based antimicrobials (E). dCas9 refers to a catalytically dead Cas9. *Nonfunctional nuclease domains RuvC and HNH. RNA polymerase (RNAP).

to degrade or inactivate the antibiotic, and the modification of essential cellular functions to prevent inhibition or binding by an antibiotic.[12] These antibiotic resistance mechanisms are commonly acquired by horizontal transfer of plasmids and other mobile elements, which have obtained resistance genes from antibiotic-producing soil organisms.[12,13] However, they can also be gained by mutation, often in genes related to the antibiotic mode of action.[13]

Horizontal gene transfer occurs via conjugation (plasmids), via transduction (bacteriophages), and via transformation (spontaneous uptake of bacterial DNA released by dying organisms followed by integration into the acceptor genome).[13,14] Plasmids harbor resistance genes and distinctive origins of replication, which allow them to initiate the replication process separately from the host genomic DNA. Multiple plasmids can be present in a given bacterium.[12] Bacteriophages can transfer genetic material containing resistance genes during their bacterial infection life cycle. Studies demonstrated that non-human-associated viromes are a vast reservoir of antibiotic-resistance genes (ARGs).[15] On the other hand, conjugative transposons, integrative and conjugative elements (ICE), and integrons (all containing resistance genes) can be acquired through conjugation or by transduction. Transposons can integrate and move to different genomic sites within a cell by means of transposases or recombinase enzymes.[16,17,16] As a consequence, one of the promising approaches that cleaves bacterial DNA or inhibits expression of critical genes is the use of the CRISPR-Cas systems.

The CRISPR-Cas system was first described in 2007 as a complex bacterial defense mechanism, or immune system, against viruses and plasmids.[18] Subsequently, substantial efforts have been made to adapt this system into a powerful gene editing tool with broad application for several organisms.[19−21] In general terms, this bacterial immune system makes copies of the invader pathogen genome and incorporates them into the CRISPR loci as spacer sequences with the help of specific Cas proteins (e.g., Cas1 and Cas2).[19,22] These spacer sequences are separated by repetitive sequences, forming an array of spacers and repetitive sequences. Short CRISPR RNAs (crRNAs) are then transcribed containing the full or partial spacer sequence.[22] Finally, both the Cas nucleases and crRNA targeting sequence associate into a cleavage complex and induce a specific lethal cut in the invader's genome, thus eliminating the invader and preventing bacterial death.[23] Depending on the molecules involved and the *cas* operon distribution, the CRISPR-mediated immune mechanism can be classified into three main types: I, II, and III.[20,22] Nevertheless, other types and subtypes of CRISPR-Cas systems are constantly described in the literature as new studies broaden

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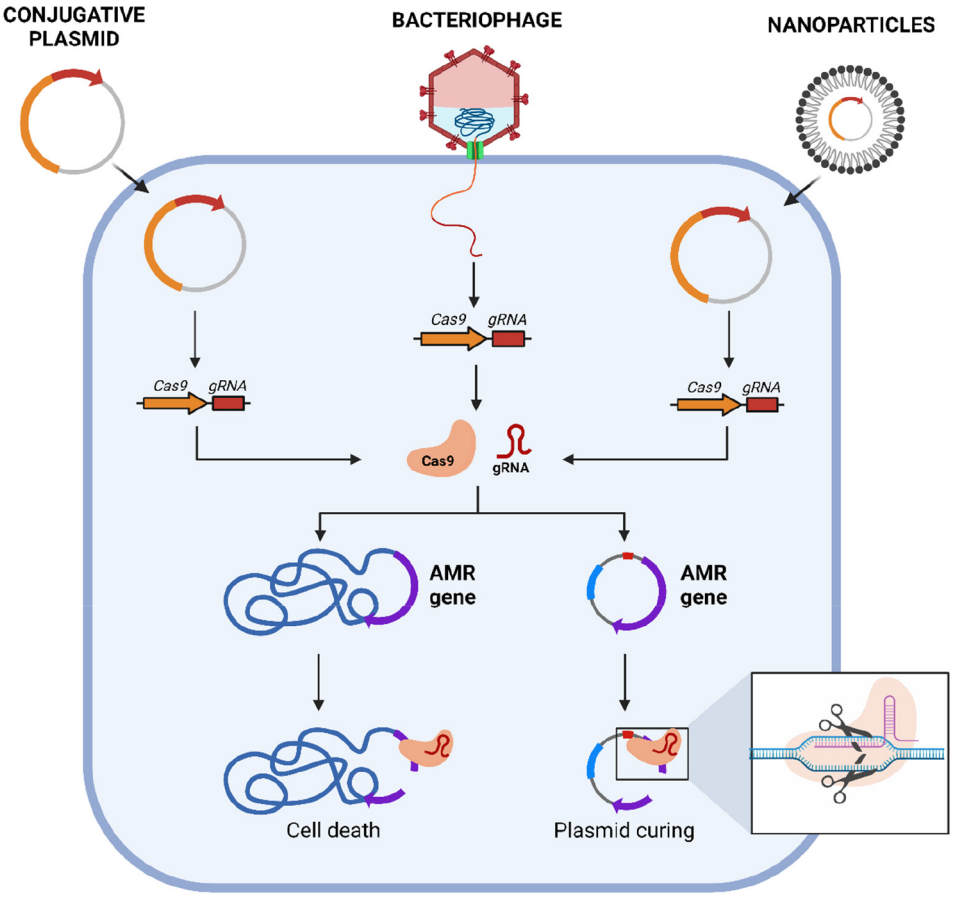

Figure 2. CRISPR-Cas system antimicrobials: mechanisms of action and delivery.

the field. Here we describe CRISPR-Cas type I, II, III, and VI as these have been used in the development of CRISPR-based antimicrobials (Table 1).

The Type I CRISPR-Cas system identified in *Escherichia coli* includes the CRISPR-associated complex for antiviral defense (Cascade) and Cas3.[19,24] The cascade complex subunit Cas6 recognizes an eight-nucleotide sequence upstream of the spacer sequence and releases a crRNA. crRNA associates with Cascade and directs the complex to its target DNA cleavage site[19,25] (Figure 1-A). On the other hand, the Type II CRISPR-Cas system, derived from *Streptococcus pyogenes*, is based on the recognition of a genome's specific dsDNA sequence by a gRNA, which recruits a Cas9 nuclease (to form a ribonucleoprotein complex) that will introduce a DSB.[25,26] This gRNA is formed by a crRNA and a trans-encoded crRNA (tracrRNA), which is complementary to a repetitive sequence. Subsequently, this RNA duplex is cleaved by RNase III, leaving the Cas9 in a complex with both tracrRNA and crRNA[23,25] (Figure 1-B). Efficient cleavage by type I and II systems require complementarity of the first eight (type I), six to eight (type II) nucleotides of the target DNA and the crRNA. Also, a protospacer-adjacent motif (PAM) next to the target sequence must be present.[12] Given the importance of these regions, mutations could result in a pathogen escaping the CRISPR-mediated immunity.[23] The type III CRISPR-Cas system can be divided into two subtypes,type III-A (*Staphylococcus epidermidis*) or III-B (*Pyrococcus furiosus*), which recruit Cas6 and, similarly to type I CRISPR-Cas, give origin to crRNA.[22,25] This crRNA is further processed at the 3′ end by Cmr or Csm effector complexes. In contrast to the other systems, type III requires transcription of the target sequence and cleavage of the DNA (type III-A) and RNA (type III-B) target[27−29] (Figure 1-C). Finally, type VI CRISPR-Cas contains Cas13 enzymes and targets single-stranded RNA (ssRNA) instead of DNA. The particularity of these Cas13 enzymes is the ability to process pre-crRNA to its mature form and to perform the degradation of a target ssRNA.[30] However, the degradation only occurs when the Cas13-crRNA complex binds to the target ssRNA[31] (Figure 1-D). Interestingly, evidence suggests that the pre-crRNA can be enough to recognize the target ssRNA, and therefore, crRNA is not a key player in this type of system.[32]

CRISPR-Cas: Designing Novel Antimicrobials. CRISPR-Cas-based technology has transformed genome engineering, previously limited to the use of specialized custom DNA-binding proteins, by benefiting from the specificity of the interaction between the Cas protein and an easily malleable target-specific gRNA complex.[33] As a result, the CRISPR-Cas system is now celebrated as a highly revolutionary genome-editing tool with several potential applications.[34] Remarkably, the discovery of the type II CRISPR-Cas9 systems and the development of related easy-to-use toolsets have opened the door to a wide range of applications for both fundamental scientific research and future clinical therapeutics.[35−37] These target-specific dual gRNA complexes rely on the interplay between two well-characterized types of RNA: the crRNA (CRISPR RNA) and the tracrRNA (Trans-activating CRISPR RNA).[31] Moreover, *Streptococcus pyogenes*-derived Cas9 (SpCas9) is not only the most commonly used nuclease for genome editing at the moment,

https://doi.org/10.1021/acsinfecdis.2c00649
ACS Infect. Dis. 2023, 9, 1283−1302

but it was also the first nuclease to be applied outside of prokaryotic cells and repurposed for genome editing in mammalian cells.[10,38,39]

In the past few years, the CRISPR-Cas system has also emerged as a promising strategy for designing new-generation antimicrobials that can target specific bacterial populations.[9,40] This offers an excellent opportunity to fight antibiotic resistant pathogens by cleaving their DNA or by selectively inhibiting genes involved in resistance, pathogenicity, or virulence.[41] As seen in Table 1, some of the most commonly targeted genes are related to antibiotic-inactivation enzymes like beta-lactamases (*bla* genes)[42] as well as genes related to membrane structure and cell permeability (*cmr-1* gene).[43] The simultaneous use of multiple gRNAs has also been implemented to target complex antibiotic resistance mechanisms like the AcrAB-TolC efflux pump system (*AcrA*, *AcrB*, and *TolC* genes) in *Escherichia coli*.[44]

The existing body of literature has described three primary mechanisms of how CRISPR-Cas can mediate antimicrobial activity, these include double-strand chromosomal cleavage, global RNA degradation, and inhibition of essential gene expression in the target bacteria.[45−47] One of the first attempts to create a CRISPR-Cas antibacterial agent used the well-known CRISPR-Cas9 nuclease system (Type I) to induce specific chromosomal DSBs and reported potent bacterial killing by targeting diverse locations throughout the genome.[48] It is important to note that the induction of plasmid DNA cleavage does not lead to host bacteria death but can be applied deliberately to eliminate resistance within the target population.[49] A different strategy proposes the use of CRISPR-Cas13 systems (Type VI) due to its promiscuous ssRNA cleavage activity and subsequent bacterial RNA degradation which can eventually halt bacterial growth.[47,50] Additionally, CRISPR-Cas antimicrobial studies have also shown an inclination toward the use of optimized CRISPR interference (CRISPRi) approaches (Figure 1-E) as opposed to traditional gene inactivation routes. The CRISPRi system was first derived from a catalytically inactive Cas9 protein, which causes transcription blockage.[51] This approach can be repurposed to repress gene expression of important genes involved in antimicrobial resistance (AMR) and pathogenicity.[44,52] Ultimately, most of these studies have still favored the CRISPR-Cas9 mechanisms over others to design their antimicrobial system *in vitro* and *in vivo* (Table 1).

The literature has demonstrated that CRISPR-Cas-based strategies can enact antimicrobial effects by two approaches: induce bacterial death or cure antibiotic-resistance-expressing plasmid.[9] The outcome of the system is determined by the location of the target DNA as CRISPR-Cas cleavage of chromosomal sequences can lead to the death of the target bacteria while targeting a plasmid-bound sequence leads to plasmid loss from the host cell (Figure 2).[48,74,75] Ultimately, it is fundamental to consider the delivery mechanisms involved in the construction of CRISPR-Cas antimicrobials as they should be able to reach virtually all bacteria within the target population.[76] The three main CRISPR-Cas vehicles explored in recent research to combat AMR bacteria are bacteriophages, nanoparticles, and conjugative plasmids (Figure 2).

CRISPR-Cas SYSTEM DELIVERY

Bacteriophages. The use of bacteriophages for infectious diseases treatment has been considered almost as soon as they were first described in 1915.[77,78] However, the initial exploration and application of phage therapy were quickly overtaken by the discovery of antibiotics after they were successfully introduced during World War II.[79] Nevertheless, research in phage therapy did not fully recede as a number of research groups from institutions like the Eliava Institute and the Hirszfeld Institute continued research on the therapeutic use of phage cocktails.[80]

The natural life cycle of bacteriophages requires replication inside a bacterial host, and they possess inherent bacteria-specific targeting and killing machinery which pose an attractive tool for the treatment of bacterial infection.[81] Bacteriophages are by far the most abundant microorganisms on the planet, and they can be found in a variety of environments where bacteria are present.[82] Bacteriophages can also be classified into two main groups according to their establishment and release mechanisms: virulent (host cell lysis) and temperate phages (stay latent inside the host).[81] Remarkably, bacteriophages also had great importance in the discovery of the CRISPR system and its function as a primitive bacterial adaptive immune system via CRISPR-Cas nucleases.[79,83]

In general, viral therapy has been extensively explored in medical research, and several fields like oncology have made promising advances regarding oncolytic virus treatments.[84] Excitingly, in 2015 the first oncolytic virotherapy was approved by the U.S. Food and Drug Administration (FDA) for melanoma treatment, and several other candidates are undergoing the latest stages of clinical trials.[85,86] The safe use of bacteriophage therapy in human patients may not be inconceivable although the literature still highlights several considerations regarding phage design and possible limitations for future applications.[87]

CRISPR-Cas Delivery Using Bacteriophages. As explained previously, bacteriophages can be classified into virulent and temperate groups depending on their life cycle. Virulent phages inject their genome into the host, hijack the host machinery for replication, and release their progeny by host cell lysis.[88] This process involves the action of two types of proteins, holins and lysins, to perforate the bacterial cytoplasmic membrane and the bacterial cell wall, respectively.[89] Temperate phages employ a lysogenic cycle where the viral genetic material gets inserted into the host genome as a prophage and stays dormant while being replicated along with the host genome.[90,91] This type of dormant genome insertion has been associated with the acquisition of antibiotic-resistance genes and virulence factors, which has prompted researchers to suggest lytic phages a more suitable vehicle for bacteriophage therapy.[92]

Classic bacteriophage therapies often take advantage of previously existing bacteriophages which can be isolated from the same environment where the target pathogen resides naturally.[93] The literature suggests that bacteriophages capable of targeting clinically associated bacteria could be isolated from hospital wastewater containers or sewage.[94] Selection for suitable phages then involves a sterilization step where the sample gets cleared of all external microorganisms and then the solution gets plated into a culture of the targeted bacteria *in vitro* to assess the formation of plaques.[95] After initial isolation and selective assays, these phages are characterized and genetically sequenced to enter the library of prospective therapeutic applications.[95] Remarkably, it is also important to characterize their interactions and possible inflammatory effects on human cells and tissues.[96]

https://doi.org/10.1021/acsinfecdis.2c00649
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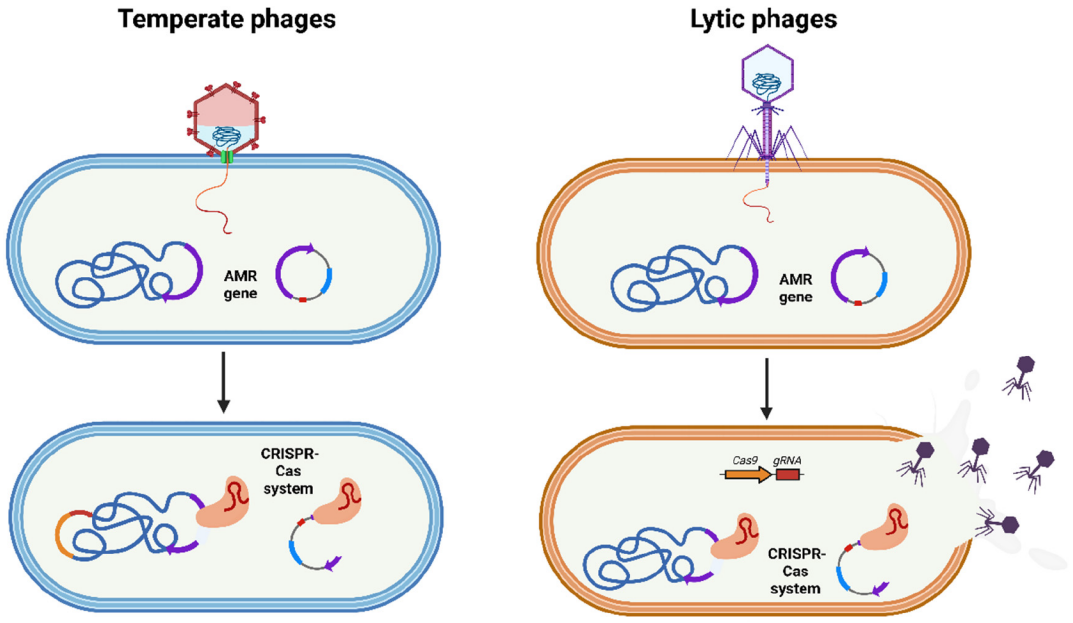

Figure 3. Bacteriophages for CRISPR-Cas9 delivery.

Recently, the incorporation of antimicrobial payloads, such as CRISPR-Cas nucleases, has become a staple approach to enhance traditional phage-based therapies.[97] The literature has extensively illustrated how both lytic and temperate phages can be used for CRISPR-Cas delivery in target bacteria (Figure 3).[98] Nevertheless, the complexity related to phage genome manipulation should not be underestimated as it demands suitable engineering tools as well as knowledge about secondary morphological and functional repercussions of such changes.[99−101] The main challenge for phage genetic engineering is related to space limitations inside the capsid which can hinder the addition of large DNA fragments like a CRISPR-Cas system without disrupting the packaging and delivery of the genome.[98] Notably, in 2015, an innovative dual system was designed to increase the efficiency of AMR *E. coli* treatment by employing both temperate and lytic bacteriophages coupled with CRISPR-Cas targeting.[73] The selected CRISPR-Cas3 system was paired with resistance to lytic phages and successfully introduced into temperate bacteriophages by removing nonfundamental accessory genes as a way to overcome the capsid space limitation. Hence, the CRISPR-Cas system only acted as a resensitization tool against conventional AMR while conferring a selective advantage to the strains that accepted resensitization. Bacteria that fail to accept the CRISPR-Cas resensitization were immediately killed by the engineered lytic phages introduced as a second part of this two-step phage treatment.[73]

In time, improved selection of phage models allowed the election of better-suited phages like fSABov for the introduction of a CRISPR-Cas9 system targeting methicillin-resistant *Staphylococcus aureus* (MRSA) infections *in vivo*.[59] The success of this optimization relies on the removal of major virulence genes from the phage genome which significantly reduced cytotoxicity in human peripheral blood mononuclear cells (PBMCs). In addition, this study demonstrated that the host range of their fSABov vector can be expanded by enhancing the phage tail fiber protein.[59] Additionally, some studies have also tested the suitability of using bacterial genetic mobile elements such as staphylococcal pathogenicity islands (SaPIs) packaged inside helper delivery phage capsids.[61] Two antimicrobial CRISPR-Cas9 modules (CRISPR-Cas9 bactericidal and CRISPR-dCas9 virulence-blocking modules) were introduced in exchange for SAPIs toxigenic genes. Moreover, the deletion of the SaPI capsid morphogenesis genes provided an additional 30 Kb packaging space which enabled the prospective insertion of additional killing/inhibition modules to counteract potential bacterial resistance. Subsequent *in vivo* assays yielded promising results in mice infected with *S. aureus* during a murine subcutaneous abscess model and a peritoneal lethal infectious model.[61] Ultimately, CRISPR-Cas3 bacteriophage antimicrobials also found success in a study targeting *Clostridium difficile* both *in vitro* and *in vivo*.[63]

Limitations. The major limitation of the use of bacteriophages as delivery vehicles is the variability in infection susceptibility between different bacterial strains.[102] This means that one phage vehicle can only target a reduced range of bacteria because their affinity depends on specific receptors on the bacterial membrane surface.[103] A proposed solution is the amplification of the host range by using "phage cocktails" where different bacteriophages are combined to increase the delivery rate of the antibacterial system.[104−106] Another limitation can be a reduced phage survival rate at the administration site (*in situ*) as many bacteriophages isolated from the environment are not equipped to survive inside a human host.[107] To avoid this, bacteriophages should undergo a rigorous selection process and can also be genetically modified to survive the immune response of the human host.[104] Phages are also susceptible to other environmental factors like temperature variations, pH, organic substances, and mechanical stress among others.[108,109] Phage encapsulation is one common protection mechanism employed for prospective therapeutic phages (it provides a platform for phage storage and increases the phage survival rate until it reaches its target location).[110] The literature has demonstrated that encapsulation enhances the adsorption and distribution of phages by enabling them to evade immune responses, withstand stomach acidity, and resist enzymes and free radicals found in tissue fluids, thereby increasing their circulation time.[111−113] Enteral

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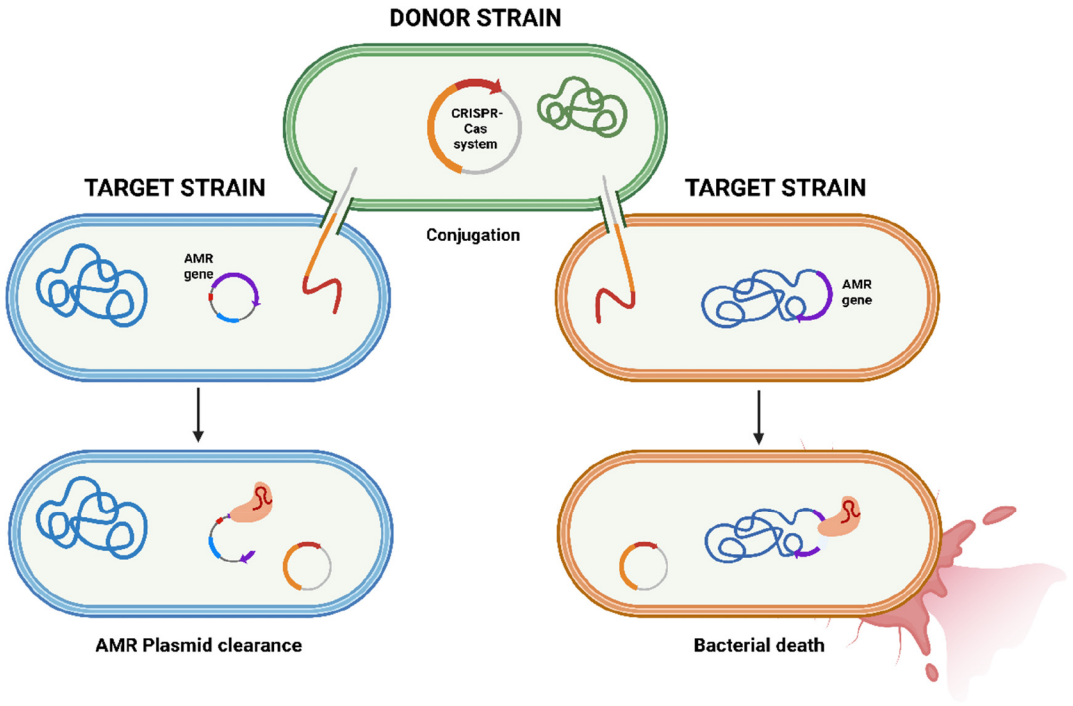

Figure 4. Plasmid conjugation for CRISPR-Cas9 delivery.

administration is the most common, but some other administration routes involve topical administration, inhalation, or injections.[114]

Safety-of-use concerns have also been raised as replicative phages could also acquire host mobile genetic elements (MGEs) which could lead to the subsequent spread of virulence genes.[115] In order to prevent contamination, some studies have opted for virulence-factor gene deletion from the host strains used in bacteriophage amplification.[59] Ultimately, all these are questions that require further research before bacteriophage can enter a clinical application phase.

Conjugative Plasmids. Several limitations regarding the use of bacteriophages and other synthetic vehicles have prompted researchers to seek more bacteria-compatible delivery routes. Research studies have assessed the use of conjugative plasmids as engineered mobile elements to transport genetic information and deploy desired functionalities within a bacterial population.[116] Plasmid sequences are highly programmable as there is a wide range of molecular biology tools which can turn them into relatively simple platforms for bacterial genetic engineering *in situ*.[117] Conjugative plasmids are circular genetic sequences that are capable of transferring from one bacterial cell to another through a process called conjugation.[118] Conjugative plasmids contain the necessary genetic information for their own transfer and can also cotransfer nonconjugative plasmids with appropriate *oriT* sites.[119,120] Briefly, the bacterial conjugation mechanism generally comprises the involvement of a type IV secretion system (T4SS) for the mobilization of DNA between a donor and a recipient cell in close physical proximity with a variety of transfer efficiencies.[55,118] Notably, several studies have demonstrated that transfer of CRISPR-Cas systems between different species of bacteria can be achieved using this transfer mechanism (Figure 4).[116,121,122] Therefore, it is not surprising that conjugation is proposed as a natural delivery mechanism for CRISPR-Cas-based antimicrobials, which can benefit from the transfer efficiency of native conjugative plasmids.[75]

CRISPR-Cas Delivery Using Conjugative Plasmids. Genetically distinguishable plasmids cannot coexist stably inside the same host cell, which has prompted researchers to classify them into Incompatibility (Inc.) groups.[123] Some studies have also uncovered the underlying mechanisms responsible for the entry exclusions that prevent the access of Inc. plasmid groups.[124] These inherent incompatibility properties suggest that plasmids utilized in synthetic biology with prospective clinical applications should be uncommon within the targeted microbial population.[125] However, plasmid screening within heterogeneous populations can be very demanding and time-consuming. One of the first attempts to develop a CRISPR-Cas antimicrobial conjugative plasmid used an Inc.P plasmid, one of the rarest type of plasmids conferring resistance in Extended Spectrum Beta-Lactamase (ESBL) strains, for targeting *blaTEM* and *blaCTX-M* beta-lactamase genes within an *E. coli* population *in vitro*.[116,126] Further studies have also effectively targeted *blaTEM-related* genes in laboratory *E. coli* strains and Enterobacteriaceae clinical isolates.[68]

An important question to consider for plasmid-driven delivery methods is that the CRISPR-Cas conjugative plasmid requires a host cell that can act as a donor toward target strains. Therefore, some research groups have suggested the delivery of this system inside well-known probiotic strains.[54] In 2021, a study achieved high transfer efficiency and treatment effectiveness using a TP114 plasmid carrying a CRISPR antimicrobial system delivered by the probiotic *E. coli* Nissle 1917.[54,127] In this model, a previously known TP114 plasmid was optimized via accelerated laboratory evolution to target AMR *E. coli* and *Citrobacter rodentium* with impressive results after 4 consecutive days of oral treatment in mice.[54,55]

The use of narrow host range plasmids such as pheromone-responsive plasmids (PRPs) has also been explored for the delivery of CRISPR-Cas antimicrobials within their specific

https://doi.org/10.1021/acsinfecdis.2c00649
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target population: *Enterococcus faecalis*.[56,57] PRPs exhibit a naturally high conjugation frequency, the ability to infiltrate and propagate within intestinal *E. faecalis* without antibiotic selection, and a competitive advantage via bacteriocin-mediated activity.[57,128−130] In 2019, a study used PRP pPD1 to target multi-drug-resistant *E. faecalis* both *in vivo* and *in vitro* and uncovered the striking difference in the effectiveness of the *in vitro* model versus the *in vivo* intestinal mouse assay.[57] The CRISPR-Cas system was able to effectively block the target plasmid dissemination within the intestinal mouse model while displaying a lower antiplasmid activity during *in vitro* assays. These differences have been attributed to several variables affecting the CRISPR-Cas system activity during *in vivo* settings such as planktonic vs biofilm lifestyles, donor-to-recipient ratio, and assay sampling time points.[57] The same year, an associated research group found promising results by selectively depleting the erythromycin-resistant enterococcal population of a murine intestine model by several orders of magnitude.[56] These results also demonstrated that a plasmid-born CRISPR-Cas antimicrobial can protect the donor strains from the uptake of the targeted antibiotic resistance. However, this study also detected perturbations in the effectiveness of the CRISPR-Cas antimicrobial after it was translated into gnotobiotic mice. Similarly to the previous study, these findings suggest that the CRISPR-Cas system benefits from the presence of microbial complexity and other external factors found in natural conditions.[56]

Research has also been directed toward the development of community-wide genetic modification tools *in situ* by engineering the mobilome. One study demonstrated that a range of replicative (mobile plasmids) and integrative (Himar transposon) vectors can exert *in situ* genetic modifications of the gut mouse microbiome with precise genetic payload.[131] Results obtained in this study also uncovered the challenges faced by probiotic donor strains to establish long-term stability within complex microbial communities. Consequently, the optimization and use of native gut bacteria as plasmid donors have been proposed to ensure the persistence of the donor strain *in situ* without the aid of antibiotic-mediated microbiota clearance.[131] More recently, the combination of CRISPR-Cas systems and transposon-mediated DNA delivery has been proposed to conduct species- and site-specific genome modifications.[132] This novel arrangement demonstrated that MGEs can also be optimized to improve the specificity and dissemination of CRISPR-Cas systems within microbial communities.[132] On the other hand, a number of studies have also strived to enhance the efficiency of current CRISPR-Cas antibiotic mechanisms by developing an active self-copying mechanism denominated pro-active genetics (ProAG).[69] In this way, the gRNA cassette is flanked with homologous sequences to the target cleavage sequence of a high copy number plasmid. The ProAG gRNA will get copied into the high copy number plasmid leading to two scenarios: amplification of gRNA expression and inactivation of the AMR sequence. The results from this study show that this ProAG system improves the inactivation of the target plasmid by ∼100-fold in comparison to the classic CRISPR-Cas-based antiplasmid methods.[69]

Overall, the use of conjugative plasmids as delivery vehicles holds great promise for a variety of engineering purposes. During conjugation, the donor strain shares the plasmid as a single-stranded DNA (ssDNA) which is subsequently complemented within the recipient bacterium with compatible host-specific modifications.[133] This could potentially facilitate the transfer and acceptance of conjugative plasmids into diverse bacterial species without prior knowledge of the specific features of those bacteria. Another advantage of conjugative plasmids is their compatibility with a wide range of bacteria, not limited by receptor recognition unlike bacteriophages.[54] Ultimately, the co-joint use of CRISPR conjugative antimicrobials and bioengineered donor strains can help circumvent the disadvantages that bacteriophages face *in vivo* when the target bacteria resides inside harsh environmental conditions (low pH, gastric fluid, proteases, etc.) such as the gut.[54]

Limitations. The first straightforward limitation toward the clinical application of conjugative plasmids is their dependence on a donor vector (often a probiotic bacterial strain) that needs to be introduced into the patient to replicate, transport and conjugate the plasmid module. Therefore, conjugative plasmids are limited by targeted population traits (planktonic vs biofilm formation) as well as the robustness of the donor strain to thrive and establish long-term colonization in different environments like skin (external) or intestinal (internal) sites of infection.[121,134] Additionally, the application of living donor vectors is limited to the gastrointestinal tract and external locations as it can be detrimental to inoculate these microorganisms into noncolonized tissues. Another very important consideration is the risk of acquisition of AMR-related genes into the plasmid vector system as the literature has extensively described the role of plasmids in the horizontal gene transfer of AMR genes among unrelated bacteria.[135] At the moment, a couple of studies have shown that the presence of the CRISPR-Cas system can immunize the donor bacteria to the acquisition of the target AMR gene, but this pressing issue requires further investigation.[56] The use of conjugative plasmids as delivery vehicles is also limited by their naturally low transfer rate, prompting numerous investigations aimed at enhancing the transfer efficacy of their selected plasmid vectors.[54] In addition, it is well documented that plasmid acquisition and maintenance enforce a high metabolic cost to the donor cell which can hinder their metabolism, growth, and reproduction.[136] Bacterial hosts are often prompted to lose nonadvantageous plasmids after exposure to nutrient scarcity, harsh environmental conditions, or competition.[137] This opens the question about how disadvantageous harboring an entire CRISPR-Cas system can be for the probiotic donor strain and how it can affect its survival rate under natural conditions. It could add a layer of complexity in the development of these vehicles as the donor strain will also be required to undergo an optimization process before it can be suitable for clinical applications.

The efficiency of plasmid transfer within bacterial biofilms has also been strongly debated in the literature.[138] It is known that close proximity of bacterial communities can be beneficial for horizontal gene transfer and the spread of MGEs like plasmids.[139,140] Nevertheless, several studies have brought attention to a disparity of plasmid distribution among different sections of a biofilm population.[141,142] The highest rate of plasmid transfer appears to be localized in the biofilm interfaces where bacteria are still undergoing replication and high metabolic activities.[143,144] Considering the mechanism of action for CRISPR-Cas antimicrobials, these unequal distributions may impair the reach of their antimicrobial effects and severely limit their use.

https://doi.org/10.1021/acsinfecdis.2c00649
ACS Infect. Dis. 2023, 9, 1283−1302

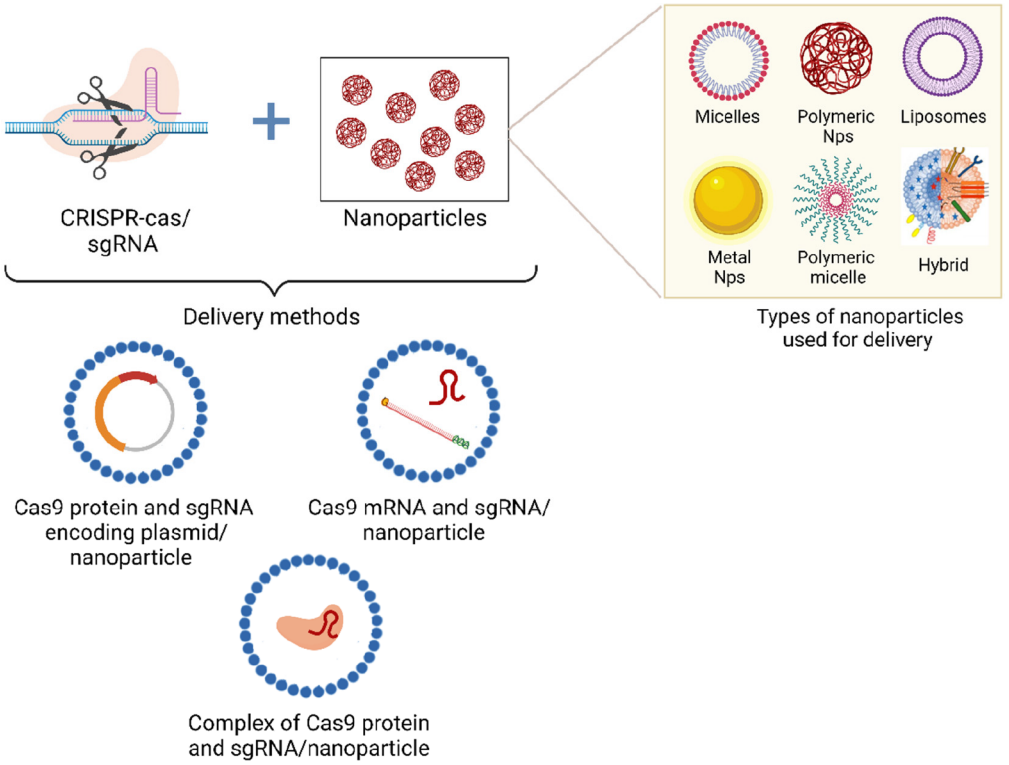

Figure 5. Nanoparticles for CRISPR-Cas9 delivery.

Lastly, the target bacteria can still harbor a plasmid from the same Inc. group as the conjugative plasmid vehicle. This can trigger what is known as plasmid competition between the resident plasmid and the CRISPR-Cas module.[145] In addition, it is important to consider that bacteria possess several mechanisms (detailed in the following sections) that could inactivate the CRISPR-Cas sequence within the genetic module. Consequently, this can lead to the creation of a defective, but still viable and replicating, CRISPR-Cas plasmid which could hinder the entrance of nonimpaired peers into the target cell.

Nanoparticles. Nanotechnology, through nanomaterials, offers an alternative to improve antimicrobial therapy and gene delivery.[146] Nanoparticles are delivery enhancers for antimicrobial agents and novel antimicrobial material distinct from conventional drugs.[147] Generally, promising nanoparticle-based antimicrobials operate under two main mechanisms against bacteria: (i) alteration of membrane potential and integrity and (ii) induction of oxidative stress through the generation of reactive oxygen species (ROS) catalyzed by nanoparticles.[148] Nanoparticles have a large specific surface area due to their small size, which enhances their reactivity, contributes to their distinctive physical and chemical properties, and renders them highly efficient in eradicating bacteria.[149] Furthermore, their nanosize-related properties allow them to penetrate deep into bacterial cells and damage their internal components, contributing to their antimicrobial activity.[149] Several attempts have been made to improve nanoparticle design by incorporating surface recognition elements such as ligands or phage-derived receptor binding proteins which enable them to selectively identify and bind epitopes on the bacterial membrane.[150] In addition, a frequent approach in the field of nanoparticle biorepatterning involves the customization of nanoparticles using aptamers or specific antibodies that exhibit high affinity toward diverse bacterial elements.[150,151] Ultimately, the interaction between nanoparticles and bacteria can also be influenced by electrostatic forces.[152] As demonstrated in recent studies, nanoparticle-based antimicrobials can be tailored with biodegradable cationic polycarbonates, enhancing the electrostatic interaction between the nanomaterials and bacterial membranes and considerably improving antimicrobial activity.[153]

In addition to their antibacterial potential, nanoparticles can fight infections by carrying drugs to deliver antimicrobial agents. Antibiotics alone may present limitations such as poor penetration into bacterial infection sites, low drug bioavailability, antibiotic-related side effects, and antibiotic resistance.[154,155] In this sense, polymeric nanoparticles have been considered a valuable tool to improve the efficacy and safety of classic antibiotics. Wrapping antibiotics in polymeric nanoparticles can enhance the solubility of hydrophobic drugs and increase the antibiotic concentration at the infection site.[155] Additionally, polymeric nanoparticles can provide a controlled release of antibiotics, which may help reduce the frequency of dosing needed and improve the safety of treatment.[156,157]

CRISPR-Cas Delivery Using Nanomaterials. Nanoparticles can be used not only to deliver drugs but also to deliver genes.[146] Genetic elements face several difficulties during target cell entrance due to serum protein adsorption, rapid clearance into the bloodstream, phagocyte uptake, inability to escape the endosome, lack of targeting ability, and the toxicity induced by the immune system, limiting their use in medical treatments.[155] To overcome this drawback, delivery systems such as metallic nanoparticles, lipid nanoparticles, and polymeric nanoparticles are under study. They have generated significant interest among researchers as protective envelopes from degradation in biological fluids by nucleases and

https://doi.org/10.1021/acsinfecdis.2c00649
ACS Infect. Dis. 2023, 9, 1283−1302

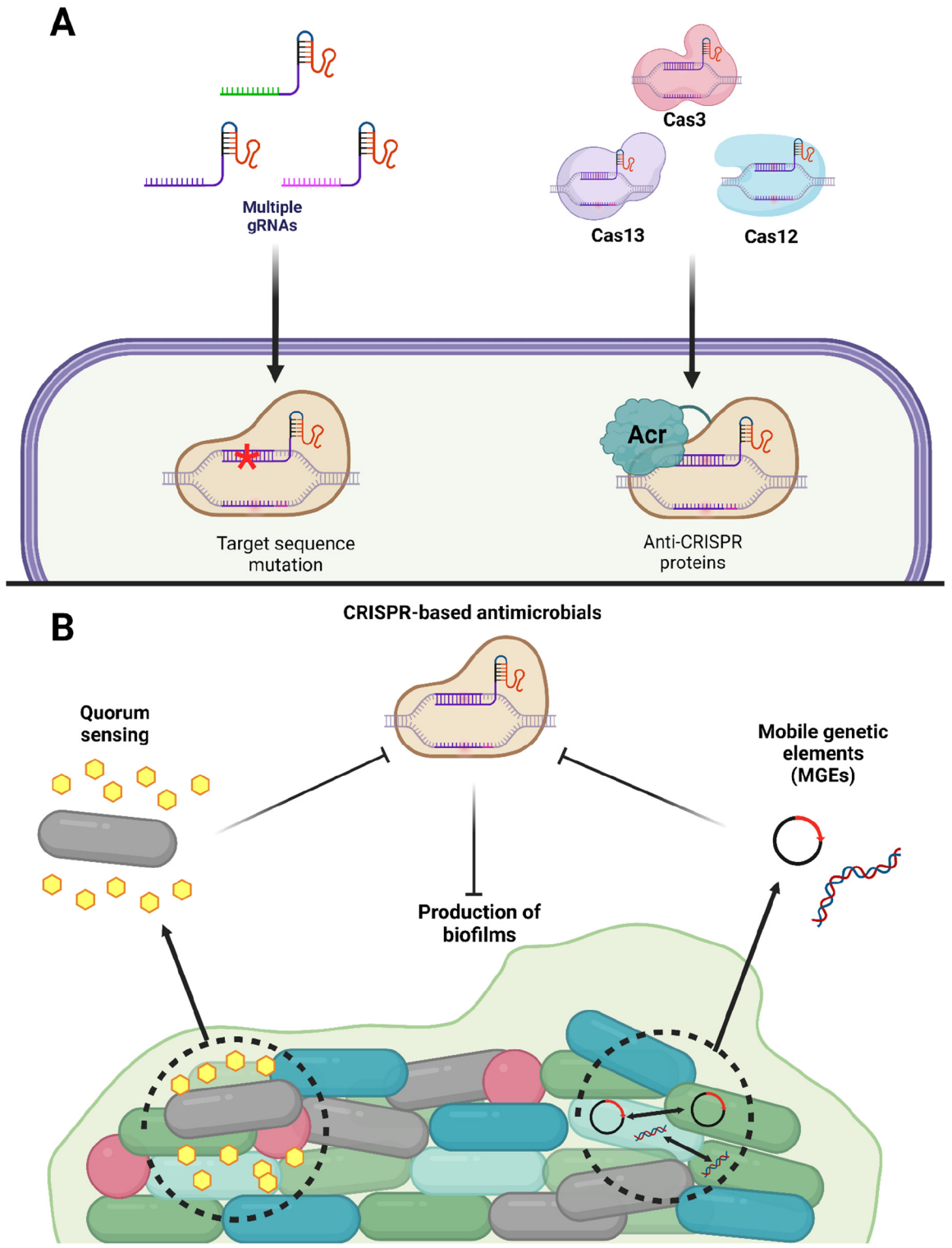

Figure 6. (A) Mechanisms of resistance toward CRISPR-Cas. (B) Bacterial community responses interfering with CRISPR-Cas antimicrobials.

proteases for fragile sequences like Cas9, mRNA, and sgRNA.[158−160]

Although CRISPR-Cas9 has the potential to knock out antibiotic resistance genes, the delivery system is inefficient.[161] For this reason, a number of strategies for nanomaterial-mediated delivery of CRISPR-Cas9 systems have been developed, which include (i) Cas protein and sgRNA encoding plasmid, (ii) Cas9 mRNA and sgRNA, and (iii) complex of Cas9 protein and sgRNA (Figure 5).[148] In addition, the physicochemical characteristics of the nanoformulation are essential for its targeting and release. For example, nanoparticles can be chemically modified to add functional groups that stabilize Cas9 and mRNA, protecting it from degradation and granting high target specificity.[159,162] The nanoparticle surface can also be conjugated with cell-penetrating peptides (enhancing cellular uptake) and/or nuclear localization signal peptides (for internal delivery).[155] Branched polyethylenimine has been used to form nanocomplexes with spCas9 and

https://doi.org/10.1021/acsinfecdis.2c00649
ACS Infect. Dis. 2023, 9, 1283−1302

facilitate the delivery and binding between the nanocomplex and the cell wall of AMR bacteria.[72]

Lipid nanoparticles are one of the most explored systems for CRISPR delivery. Lipid nanoparticles can be used to encapsulate negatively charged CRISPR plasmid DNA and mRNA, guiding and protecting the mRNA during its cell membrane diffusion.[163,164] Polymeric nanoparticles have been favored for CRISPR delivery due to their low immunogenicity and their high compatibility with living tissues.[165,166] Furthermore, metal-based nanoparticles have also been considered suitable for delivering ribonucleoprotein (RNP) complexes.[155] For example, the design of a polymer-derivatized Cas9 and a gRNA nanocomplex (Cri-nanocomplex) displayed significant inhibition of MRSA growth in the presence of methicillin by altering the *mecA* gene.[72] In another study, carbon quantum dots covalently conjugated to Cas9 and papG-targeted gRNA were employed to form a Cri-dot-papG nanocomplex for the delivery of a CRISPR-Cas antibiotic in Uropathogenic *E. coli* (UPEC). This system targeted the gene associated with the fimbrial adhesion virulence factor (*papG* gene) and registered a significant decrease in *papG* expression, thereby reducing the pathogenicity of UPEC.[53]

Limitations. For proper Cas mechanism and protein expression, accurate delivery of the CRISPR-Cas system is critical. However, there are several challenges at the *in vivo* level, such as the immunogenicity caused by the vector[160] or the efficient packaging and localization of the CRISPR-Cas components using nanoparticles. The risk of immunogenicity associated with CRISPR-Cas9 gene editing highly depends on the delivery route used.[160] However, some CRISPR-Cas-based therapies incorporate the protein within biocompatible nanoparticles to reduce immunogenesis.[167,168] For example, vectors based on lipid nanoparticles may offer a viable alternative for *in vivo* applications since lipids are less immunogenic than other vectors, such as viruses.[169] However, there needs to be more knowledge of the interaction of nanomaterials with cells and tissues. Another limitation is the efficient packaging and localization of system components, for which nanoparticles can be tailored in various ways, for example, by modifying cell-penetrating peptides and specific cell receptors to enhance protein uptake by cells and its interaction with target cells.[155] Another strategy is a surface modification with biocompatible polymers such as polyethylene glycol to reduce the elimination mediated by reticuloendothelial cells and increase the lifetime in the blood.[160] This strategy will contribute to reducing the toxicity of metal nanoparticles for drug delivery which, at low doses, are generally not toxic, but their prolonged administration can cause long-term aggregation and potential toxicity. There are still studies on the CRISPR-Cas-nanoparticle delivery system before they can be applied as delivery systems for antibacterial activity. Further investigation with *in vivo* subjects is required to determine short- and long-term toxicity.

BACTERIAL MECHANISMS OF RESISTANCE

CRISPR-Cas systems have been developed as a new form of antimicrobial defense to counteract bacterial infections. However, different studies have shown that bacteria can develop mechanisms to evade the CRISPR-Cas system's action with an overall frequency of approximately 10^{-4},[170] potentially rendering CRISPR-Cas systems ineffective as a stand-alone antimicrobial agent.

Research has shown that bacteria can acquire several mutations during the natural process of CRISPR adaptive immune responses. Occasionally a mutation may grant resistance to phages or antibiotics.[171,172] Currently, the role of CRISPR as a precision genome editing tool is widely celebrated; however, studies have shown that many newly discovered CRISPR systems are not quite precise. Type III CRISPR mechanisms demonstrated indiscriminate targeting for DNA cleavage, unlike the Type II CRISPR system, which relies on the Cas9 nuclease to cut specific DNA sequences.[173] Therefore, the rational design of CRISPR-based tools like novel antibiotics has shown strong inclinations toward using Cas9 nucleases.[174] However, the heavy reliance on these specific machinery raises concerns about future resistance mechanisms. The literature has described several risk factors, such as the spontaneous mutations in the *Cas* genes or the target sequences and the presence of the anti-CRISPR (*Acr*) genes in the target host genomes[175] (Figure 6-A). Here we describe the main mechanisms involved in CRISPR-Cas resistance by bacteria.

Escapers through Mutations. Mutations in either the CRISPR-Cas complex or the targeted genetic region can enable bacteria to evade the effects of the CRISPR-Cas system and survive. If a mutation in the first CRISPR-Cas complex to enter the bacteria occurs, the bacteria would still be sensitive to a functional system (Figure 6-A). Still, if the target region undergoes large deletions, the bacteria would effectively evade the system and become resistant.[74,176]

Research has recorded some examples of bacteria using mutations to evade the CRISPR-Cas system. In a 2018 study, *E. coli* was exposed to a phage carrying a protospacer with a specific PAM sequence and developed mutations in the PAM sequence of the target gene, rendering it unrecognizable to the CRISPR-Cas system.[177] As a result, the bacteria were able to survive and continue to replicate, while the phage was unable to infect the cells.[177] Another example of bacteria using mutations to evade the CRISPR-Cas system was observed in *Streptococcus thermophilus*, which was exposed to a plasmid that carried a protospacer with a specific PAM sequence. The bacteria were able to develop mutations in the PAM sequence of the target gene, preventing the CRISPR-Cas system from recognizing and destroying the plasmid. As a result, the bacteria were able to maintain the plasmid and continue to replicate.[176]

In addition to mutations in the PAM sequence, mutations can also occur in the spacer sequence, which is the small fragment of foreign DNA incorporated into the bacterial genome. If the spacer sequence is mutated, it may no longer match the target DNA, rendering the CRISPR-Cas system ineffective. Mutations in the spacer sequence have been observed in *Vibrio parahaemolyticus*, which was exposed to a phage that carried a protospacer with a specific spacer sequence. The bacteria were able to develop mutations in the spacer sequence, preventing the CRISPR-Cas system from recognizing and destroying the phage.[178]

A few scenarios have been described after the loss of large genetic sequences. The first can lead to positive outcomes, resulting in the inactivation of antibiotic resistance or virulence traits related to those genes.[155] Nonetheless, point mutations where the gene function is preserved can enable bacteria to escape recognition by the Cas nucleases while maintaining their pathogenic traits.[179] This issue can be solved by programming the CRISPR-Cas system to simultaneously target

https://doi.org/10.1021/acsinfecdis.2c00649
ACS Infect. Dis. 2023, 9, 1283−1302

several positions at the undesired locus. Fittingly, the natural mechanism of CRISPR immunity encompasses multiple targeting sequences, enabling easy and convenient multiplexing for antimicrobial sequence targeting.[9] Some studies, however, showed no correlation between the number of target sites and killing efficiency by assessing 10 different gRNAs with 1 to 25 target sites in an *E. coli* genome and obtaining similar results.[180] Other studies indicate that a high number of target sites can still limit the evolution of resistant strains with chromosomal mutations.[181]

In addition to the previous resistance response, it was found that the most common site of mutations was localized in the plasmid-encoded SpCas9 used to introduce the system into the target strain. Some studies described recombination and deletions that resulted in the inactivation of the CRISPR loci and the elimination of *Cas* genes and target sequences, to name a few.[48,63] A proposed solution was reintroducing an intact copy of the plasmid to circumvent this resistance mechanism.[182] Reduction of the CRISPR array to a single spacer repeat could prevent recombination between repeats and subsequent spacer deletion.[40]

One strategy for overcoming mutations in the targeted DNA sequences is to use multiple gRNAs to target different sites within the bacterial genome. This approach increases the likelihood of destroying the target DNA, even if it has mutated in one or more of the gRNA binding sites. Multiple gRNAs can be delivered simultaneously using a variety of methods, including plasmids, viral vectors, or nanoparticles.[183] Another strategy is to use CRISPR-Cas systems that target different parts of the bacterial genome. For example, CRISPR-Cas9 and CRISPR-Cas13 systems target DNA and RNA, respectively. Targeting different parts of the genome can increase the likelihood of destroying the target, even if it has mutated in one part of the genome. Additionally, using different CRISPR-Cas systems can be effective against bacteria that have developed resistance to one particular system.[184,185]

A third strategy is to engineer the CRISPR-Cas system to target regions of the genome that are less likely to mutate. For example, the CRISPR-Cas system can be designed to target conserved regions of the genome, which are less likely to undergo mutations such as the *gyrA* gene in *E. coli*. The system was effective in killing antibiotic-resistant *E. coli* strains, which had mutations in other regions of the *gyrA* gene.[186]

A fourth strategy is to use high-throughput screening to identify gRNAs that are effective against bacteria with specific mutations. In this approach, large libraries of gRNAs are synthesized and screened for their ability to target mutated sequences. A study successfully utilized a library of 1.4 million gRNAs to identify all gRNAs effective against antibiotic-resistant *E. coli* strains.[187,188]

Inactivation via Anti-CRISPR (Acr) Proteins. Anti-CRISPR proteins (Acrs) interact with essential components of the diverse CRISPR-Cas systems inhibiting the cleavage activities of the protein.[189] It has been reported that most CRISPR-Cas systems can undergo inactivation, especially in prokaryotes where Acrs are highly diverse.[190] For example, phylogenetic studies in a pool of around 600 genes belonging to a *Pseudomonas aeruginosa* strain collection found *acr*-related genes in at least 30% of them,[191] restricting CRISPR-Cas applications. The first ACR protein to be discovered was AcrF1, which was identified in a study of the Type I-F CRISPR-Cas system in *Pseudomonas aeruginosa*. Since then, many other ACR proteins have been identified in various bacterial species, including Type II-A, Type II-C, and Type V-A CRISPR-Cas systems.[191] ACR proteins function by binding to various components of the CRISPR-Cas system, such as the Cas proteins or the gRNA, and preventing them from binding to the target DNA. This inhibition can occur at different stages of the CRISPR-Cas process, including DNA binding, RNA-guided complex formation, and target cleavage.[192]

One of the most well-studied ACR proteins is AcrIIA4, which inhibits the activity of the Type II-A CRISPR-Cas system found in *Streptococcus pyogenes*. AcrIIA4 binds to the Cas9 protein and prevents it from binding to the target DNA. This inhibition is reversible and can be overcome by increasing the concentration of Cas9, suggesting that AcrIIA4 is a competitive inhibitor.[193,194] The discovery of ACR proteins has important implications for the use of CRISPR-Cas systems in genome editing and other applications. The presence of ACR proteins can reduce the efficiency and specificity of the CRISPR-Cas system, leading to off-target effects and potentially reducing the therapeutic potential of this technology.[189,195]

Anti-anti-CRISPR strategies refer to the development of tools and techniques to overcome the inhibitory effects of anti-CRISPR (ACR) proteins on CRISPR-Cas systems. These strategies are important for improving the efficiency and specificity of CRISPR-Cas systems in genome editing and other applications. One possible solution has been described as an "anti-anti-CRISPR" strategy where anti-CRISPR-associated (*aca*) genes, generally encoded at the 3′-end of *acr* gene regions, are included in the antimicrobial design as they play a role in the repression of *Acr* genes.[192,196] One approach to anti-anti-CRISPR strategies involves the development of small molecule inhibitors that can bind to and inhibit the activity of ACR proteins. These inhibitors could be used to prevent ACR proteins from binding to the CRISPR-Cas system and inhibiting its activity, thereby improving the efficiency of genome editing and other applications.[197,198] Another approach is the use of engineered CRISPR-Cas systems that are resistant to the inhibitory effects of ACR proteins. For example, mutations can be introduced into the Cas protein or gRNA to prevent binding by ACR proteins, or alternative nucleases can be used that are not affected by ACR proteins.[199]

Other Forms of Escape. Bacteria can also resist the CRISPR-Cas system by modifying their own DNA. One such modification is methylation, which involves adding a methyl group to the DNA sequence. This modification can prevent the CRISPR-Cas system from recognizing and destroying the target DNA by masking the PAM sequence required for recognition. A study found that some bacteria can methylate their DNA in the PAM sequence, preventing the CRISPR-Cas system from recognizing and destroying the target DNA.[200]

Some authors also hypothesize that *E. coli* "escapers" survive the DSBs within the chromosome when there is a low expression of SpCas9 to overcome the RecA-mediated repair.[201] In recent research, researchers choose the promoters J23116, J23111, and J23100 for weak, intermediate, and strong expression of SpCas9, respectively. The expression strength was linked to the killing efficiency of the system (Figure 6-A). They demonstrate that the level of SpCas9 is an important parameter to consider to reduce the number of cells surviving DSBs.[180]

https://doi.org/10.1021/acsinfecdis.2c00649
ACS Infect. Dis. 2023, 9, 1283−1302

IMPACT OF ENVIRONMENTAL CONDITIONS AND LIFESTYLE ON THE ABILITY OF BACTERIA TO SURVIVE CRISPR-BASED ANTIMICROBIALS

Population Variations. Microbial communities, composed of a diverse array of genera, species, and strains of microorganisms coexisting in the same environment, are characterized by complex networks of interactions where each member performs specific functions while simultaneously competing for limited resources.[202,203] Competition for resources is a fundamental driving force behind the evolution of microbial communities. In environments where resources are limited, such as in soil or in the human gut, microbial communities must compete for nutrients and other essential factors in order to survive and reproduce.[202,204] This competition can trigger a selective pressure for all the microbes suited for efficient acquisition and utilization of scarce resources, as well as those that are able to outcompete other microbes for these resources.[205] Mechanisms, such as horizontal gene transfer (HGT), which promotes genetic diversity, are critical for microbial adaptation and evolution under these conditions.[206] It is important to discuss how some of these mechanisms may restrict the use of CRISPR-based antimicrobials or limit their effectiveness against certain groups of bacteria.

Interference of Different Plasmids and Other Mobile Genetic Elements. MGEs, including plasmids, transposons, bacteriophages, and integrative conjugative elements, are important drivers of bacterial evolution and can significantly impact the effectiveness of CRISPR-based antimicrobials.[76,175,207] As mentioned previously, microbial evolution can be shaped by competition for resources, prompted by the acquisition of beneficial genes or genetic elements (plasmids and MGEs) and enabling microbes to utilize resources effectively or protect themselves against external hazards.[208,209] The ability of MGEs to facilitate the transfer of genetic information between different bacterial species allows for the evolution of novel genetic combinations and the emergence of new bacterial lineages.[210] In highly competitive environments, MGEs provide an advantage for bacteria,[209,211,212] and as a result, bacteria have evolved adaptations to restrict or reduce the action of genomic defense systems.[208]

Currently, CRISPR-based antimicrobials are not widely utilized, therefore no reports of resistance to this type of antimicrobial have been documented in clinically significant bacteria. Nevertheless, a critical concern for this type of antimicrobial is the possibility of bacteria escaping CRISPR-Cas dsDNA breakage due to mutations in chromosomal regions encoding for CRISPR-Cas effector protein, gRNA, or the target sequence,[49,62,213−215] thereby inducing resistance. Along with CRISPR resistance resulting from mutations in chromosomal genes, increasing evidence suggests that several proteins possessing anti-CRISPR activity are encoded in plasmids and other MGEs, which can promote resistance to CRISPR-Cas systems, thereby limiting their use as antimicrobial agents. In a recent study, it was identified that anti-CRISPR proteins are up to 15 times overrepresented in the MGEs of ESKAPE pathogens when compared to the "nonmobile" genome.[216] Furthermore, loci that code for anti-CRISPR proteins have been detected in MGEs of various Gram-negative bacteria,[217] some of the clinical relevance.[218] Considering the high frequency of horizontal transfer events between bacteria and the selective pressure that CRISPR-based antimicrobials could impose, it is expected that the transfer of plasmids that confer resistance to cutting by Cas nucleases will pose a challenge for the use of this type of antimicrobial therapy alternatives.

Microbial Biofilms. An adaptation mechanism employed to survive in diverse environments is the growth of biofilms, which are communities of microorganisms that form on various surfaces and are surrounded by an extracellular matrix of biopolymers.[219] These structures have several characteristics that contribute to their survival, including surface adherence protection from desiccation, chemical perturbation, invasion by other bacteria, and killing by immune cells.[220] Biofilm formation is also one of the main sources of antibiotic resistance in bacterial communities due to the poor penetration of these molecules into the biofilm matrix. Cells within biofilms are significantly more resistant to antimicrobials than their free-living counterparts as demonstrated by the literature with some studies showing up to 1000-fold increased resistance.[221] Even though the resistance of biofilms to antimicrobials is often considered a passive or nonspecific mechanism, a combination of acquired and adaptive mechanisms contributes to antibiotic resistance in these structures.[222] Genetic adaptation within biofilms allows cells to adapt to their surroundings and increase their resistance to antibiotics as shown by the expression of multi-drug resistance proteins by cells within biofilms.[223] Some antibiotics can also induce the expression of resistance-related enzymes in these cells.[224]

External factors such as the environment and lifestyle of the target bacteria can significantly impact the effectiveness of CRISPR-Cas-based antimicrobials,[180] potentially affecting the treatment of AMR-born infections. The environment where bacteria are localized can affect their growth rate, metabolic activity, and gene expression, which can further impact their antimicrobial susceptibility. Similarly, it has been extensively described that bacterial lifestyles significantly affect their ability to survive and replicate in the presence of antimicrobials.[220,225] CRISPR-based antimicrobials may face the same limitation as conventional antibiotics if they target bacteria inside biofilms as the presence of the extracellular matrix can hinder the antimicrobial penetration,[220,223] making it more difficult for CRISPR-Cas systems to reach their targets and cleave targeted DNA sequences (Figure 6-B). Additionally, bacteria within a biofilm tend to have a high degree of heterogeneity in metabolic states and gene expression,[224,226] leading to individual cells exhibiting resistance due to reduced permeability, low metabolic activity, and the production of persister cells.[220,227] As a result, current biofilm treatments often require higher doses of conventional antimicrobials administered for longer periods of time compared to the doses and duration needed to treat planktonically grown cells.[221,223]

As discussed in this section, some features of the lifestyle of microbial communities have been identified as a potential obstacle to the effectiveness of CRISPR-Cas-based systems as antimicrobial agents. Nonetheless, certain lifestyle changes could also provide opportunities for the successful deployment of this type of antimicrobial. A study demonstrated that biofilm formation significantly enhanced the conjugative transfer proficiency of a CRISPR-bearing plasmid.[121] This effect can be attributed to the proximity of cells within a biofilm community which can be advantageous to enhance CRISPR-based antibacterial killing. Despite the antimicrobial potential of CRISPR-based systems, certain bacterial strains still exhibit

https://doi.org/10.1021/acsinfecdis.2c00649
ACS Infect. Dis. 2023, 9, 1283−1302

a natural resistance to CRISPR-Cas and represent a significant challenge for CRISPR application. In light of these limitations, CRISPR-Cas antimicrobials may not be effective as standalone treatments, but instead, they could be employed as a supplementary approach to address microbial resistance.

CONCLUDING REMARKS AND FUTURE PROSPECTS

Due to the proliferation of antibiotic-resistant bacteria, the management of infectious diseases is becoming increasingly challenging. The high specificity and reprogrammability of CRISPR-Cas constructs have piqued the interest of the research community for the development of a plethora of novel applications. *In vitro* and *in vivo* studies have shown that CRISPR-Cas systems have the potential to serve as next-generation antimicrobials. Although the regulatory and ethical aspects of CRISPR-Cas-based antimicrobials and their clinical application have not been widely discussed in the literature, the general use of the CRISPR-Cas technology has raised several concerns.[228] A significant issue to consider is the risk of off-target mutations with consequent deleterious effects observed in large genome sequences such as human cell lines.[229,230] Bearing in mind that CRISPR-CAS-based antimicrobials will be in contact with the patient tissue, it is essential to anticipate and resolve these hazards during the early stages of antimicrobial design by optimizing the guide RNA target system[231] and upgrading the delivery vehicle specificity. Furthermore, the risks related to the potential release of the CRISPR-Cas system into the environment need to be assessed, and strict control regulations for therapeutic handling and application should be enforced.[231] It is imperative to engage and educate the medical and general populations regarding all distinct aspects of this new technology as well as to update legislative guidelines related to the use of gene-editing tools to ensure their well-informed and safe application.[232]

Prospective applications of CRISPR-Cas-based antimicrobials include clearance of the AMR pathogenic microorganisms or restoring their susceptibility to conventional antibiotics. However, the main challenge of using CRISPR-Cas systems as therapeutic solutions is the development of effective vectors that can deliver exogenous DNA. Currently, the most commonly employed methods for CRISPR-Cas delivery include bacteriophages, nanoparticles, and conjugative plasmids. There are several challenges and limitations that can be foreseen in the development of CRISPR-based antimicrobials. The most concerning one is the generation of resistance mechanisms by targeted bacteria. Further research is necessary to assess the true potential of CRISPR-Cas systems in more realistic microbial communities and to fully understand the risks associated with this technology.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Dr. Alexander Mueller for technical support. All the figures in this Review were created with Biorender.com.

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https://doi.org/10.1021/acsinfecdis.2c00649
ACS Infect. Dis. 2023, 9, 1283−1302