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CRISPR-Cas Systems in the Fight Against Antimicrobial Resistance: Current Status, Potentials, and Future Directions

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Background: Antimicrobial resistance (AMR) is a critical global health concern that threatens the efficacy of existing antibiotics and poses significant challenges to public health and the economy worldwide. This review explores the potential of CRISPR-Cas systems as a novel approach to combating AMR and examines current applications, limitations, and prospects.

Methods: A comprehensive literature search was conducted across multiple databases, including PubMed, Google Scholar, Scopus, and Web of Science, covering publications published from 2014 to August 2024. This review focuses on CRISPR-Cas technologies and their applications in AMR.

Results: CRISPR-Cas systems have demonstrated efficacy in combating antimicrobial resistance by targeting and eliminating antibiotic-resistance genes. For example, studies have shown that CRISPR-Cas9 can effectively target and eliminate colistin resistance genes in MCR-1 plasmids, restoring susceptibility to carbapenems in bacteria such as *E. coli* and *Klebsiella pneumoniae*. Further molecular findings highlight the impact of CRISPR-Cas systems on various bacterial species, such as Enterococcus faecalis, in which CRISPR systems play a crucial role in preventing the acquisition of resistance genes. The effectiveness of CRISPR-Cas in targeting these genes varies due to differences in CRISPR locus formation among bacterial species. For instance, variations in CRISPR loci influence the targeting of resistance genes in *E. faecalis*, and CRISPR-Cas9 successfully reduces resistance by targeting genes such as tetM and ermB.

Conclusion: CRISPR-Cas systems are promising for fighting AMR by targeting and eliminating antibiotic-resistant genes, as demonstrated by the effective targeting of colistin resistance genes on MCR-1 plasmids and their similar activities. However, the effectiveness of CRISPR-Cas is affected by variations in the CRISPR loci among bacterial species. Challenges persist, such as optimizing delivery methods and addressing off-target effects to ensure the safety and precision of CRISPR-Cas systems in clinical settings.

Keywords: CRISPR-Cas systems, antimicrobial resistance, gene editing, bacteriophage delivery

Introduction

The global healthcare landscape faces an unprecedented challenge in the form of antimicrobial resistance (AMR), which fundamentally threatens our ability to effectively treat common bacterial infections. This growing crisis represents one of the most critical public health concerns of the 21st century and is characterized by the evolution of bacteria that reduce the effectiveness of drugs intended to combat them.[1,2] The World Health Organization (WHO) and similar health bodies have emphasized the urgent need for coordinated global efforts to address the AMR crisis. Understanding the extent of

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AMR, regional trends, and key pathogen-drug combinations that drive this burden is essential. If left unaddressed, AMR may become the next pandemic, necessitating public health mediation.[2] AMR occurs when bacteria, viruses, fungi, and parasites evolve to resist antibiotics designed to kill or inhibit their growth, leading to increasingly difficult-to-treat infections. This not only increases the risk of disease transmission, severe illness, disability, and death but also poses a threat to public health and global economies.[3]

Human activities such as the inappropriate use of antimicrobial drugs in humans, animals, and agriculture have accelerated the development and spread of AMR. Despite decades of research and intervention, traditional approaches to combating AMR have proven insufficient, creating an urgent need for innovative solutions. While antimicrobial drugs are vital in modern medicine, the rise of drug-resistant pathogens jeopardizes the treatment of common infections and the success of life-saving procedures, such as surgery and cancer treatment. The impact of AMR extends beyond human health, affecting animals and plants, reducing agricultural productivity, and threatening food security.[4] The economic burden of AMR is substantial, straining healthcare systems and national economies through increased care costs and reduced productivity. This global issue is exacerbated by factors such as poor access to clean water, sanitation, and healthcare, particularly in low-resource settings.[4]

Clustered regularly interspaced short palindromic repeat (CRISPR) technology has emerged as a promising solution for this challenging landscape. This revolutionary genetic engineering tool, initially derived from the bacterial immune system, offers exceptional precision in targeting and modifying pathogen genomes.[5,6] Although traditional antibiotic development has slowed considerably, CRISPR presents a novel strategy for limiting and reducing antibiotic resistance in pathogens through targeted genetic modifications.[5,6] CRISPR has the potential to disrupt antibiotic resistance mechanisms in bacteria through the targeted inactivation or activation of specific genes. This approach could lead to the development of novel antibiotic compounds or the revitalization of existing compounds. Given the slow pace of antibiotic discovery, compounded by the challenges and lack of financial incentives in this area, CRISPR represents a novel strategy for limiting and reducing antibiotic resistance in pathogens.[6] Genome engineering techniques using CRISPR can genetically activate or inactivate sequence-specific DNA targets to combat resistance.[7,8] This review aims to comprehensively examine the potential of CRISPR genetic technology as a groundbreaking approach to combat antimicrobial resistance and to analyze current applications, limitations, and prospects. By synthesizing recent advances and challenges, we sought to clearly understand how CRISPR technology might revolutionize our approach to combat antibiotic resistance and shape the future of antimicrobial therapy.

Methodology

A comprehensive search was conducted using multiple databases, including PubMed, Google Scholar, Scopus, and Web of Science, up to August 2024. The search utilized terms such as “CRISPR-Cas systems”, “antimicrobial resistance”, “base editing”, “prime editing”, “delivery methods”, AND, OR “gene regulation”, combined with Boolean operators (AND, OR) to refine the results. The inclusion criteria were peer-reviewed articles, reviews, and clinical trials published in English between 2014 and August 2024 focusing on CRISPR-Cas technologies and their applications in antimicrobial resistance. These included studies on advancements in base and prime editing, delivery mechanisms, and gene regulation through epigenome engineering. Non-peer-reviewed articles, editorials, opinion pieces, and studies that were not published in English or before 2014 were excluded.

The Current Global Burden of AMR

Regional Trends in AMR

The impact of AMR is evident in various parts of the world, with differing prevalence rates and outcomes. In the UK, the number of patients infected with AMR increased from 61,946 in 2018 to 65,162 in 2019, highlighting the growing challenges in healthcare systems.[9] In the United States, the Centers for Disease Control and Prevention (CDC) report over 2.8 million antibiotic-resistant infections annually, resulting in more than 35,000 deaths per year in the region.[10] The burden of AMR in India is significant, with over 50,000 infants at risk of sepsis-related deaths due to antibiotic-resistant bacteria, resulting in one child dying every nine minutes from such infections.[11] These statistics highlight notable regional disparities in AMR burden, with developing countries experiencing higher mortality rates owing to inadequate healthcare and limited access to effective treatments (Table 1).

Table 1 Global Burden of Bacterial Antimicrobial Resistance*

Infection Type	Deaths with Infection	AMR Deaths	AMR DALYs	Attributable Deaths	Attributable DALYs
All	3,830,000	1,046,000	64,344,000	250,000	15,031,000
Bloodstream infections	557,000	236,000	15,512,000	56,000	3,620,000
Bone and joint infections	3,950	2,180	78,000	493	18,000
Cardiac infections	5,810	3,610	153,000	850	35,000
Gonorrhoea and chlamydia	684	NA	11,000	NA	1060
CNS infections	135,000	65,000	4,665,000	15,000	1,079,000
Diarrhoea	589,000	27,000	2,086,000	6,280	489,000
Other infections	863,000	NA	NA	NA	NA
Intra-abdominal infections	159,000	106,000	3,556,000	26,000	872,000
Lower respiratory and thorax infections	1,000,000	521,000	34,412,000	119,000	7,786,000
Bacterial skin infections	44,000	18,000	580,000	3720	120,000
Tuberculosis	369,000	42,000	1,847,000	18,000	748,000
Typhoid, paratyphoid, and iNTS	89,000	14,000	1,067,000	2270	173,000
Urinary tract infections	16,000	12,000	376,000	2790	89,000

Notes: *Data show point estimates for 2019. AMR deaths refer to deaths associated with antimicrobial resistance, whereas attributable deaths specifically indicate deaths directly attributable to AMR. AMR DALYs show disability-adjusted life-years associated with resistance, and the attributable DALYs represent the disease burden directly attributable to AMR. Deaths with Infection indicate total deaths involving each infection type, regardless of the resistance status. NA=not applicable. iNTS=invasive non-typhoidal salmonellae.[1]

Key Pathogens and AMR Prevalence

The European Antimicrobial Resistance Surveillance Network (EARS-Net) data from 2015 to 2019 revealed that the prevalence of AMR varies by bacteria, antimicrobial class, and geographical region. The key resistant bacteria included *Pseudomonas aeruginosa* (5.6%), *Streptococcus pneumoniae* (5.3%), *Enterococcus faecium* (4.5%), *and Acinetobacter spp*. (1.7%), *Klebsiella pneumoniae* (11.3%), *Enterococcus faecalis* (6.8%), and *Escherichia coli* (44.2%). The WHO,s Global Antimicrobial Surveillance System (GLASS) identified widespread AMR among 500,000 people across 22 countries, with *E. coli* and *K. pneumoniae* showing resistance rates to ciprofloxacin, an antibiotic used for urinary tract infections (UTI), ranging from 8.4% to 92.9% and 4.1% to 79.4%, respectively.[10,12] The prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) remains a significant global concern, accounting for 13–74% of *S. aureus* infections worldwide. In the United States, MRSA infections have led to an estimated 19,832 deaths from 119,247 infections.[13] These data underscore the need for enhanced infection control measures and targeted interventions to curb the spread of resistant pathogens.

Public Health and Economic Impact

The global impact of AMR extends beyond public health and poses significant economic challenges. The World Bank estimates that by 2050, AMR could lead to a loss of $1 trillion to $3 trillion in global gross domestic product (GDP) annually, along with substantial healthcare costs.[14] The Organisation for Economic Co-operation and Development (OECD) projects that resistance to last-resort antibiotics could double by 2035 compared to 2005, further exacerbating the economic burden and threatening global health security.[15] In sub-Saharan Africa, the region with the highest all-cause mortality rate is directly linked to AMR (Table 1), and the death toll is expected to increase significantly if effective interventions are not implemented. The emergence of drug-resistant fungal infections, such as *Candida auris*, and drug-

resistant parasites, particularly in malaria control, further complicates the AMR landscape, requiring urgent global attention and action.[16] The future outlook for AMR is concerning, with projections indicating that, by 2050, 10 million deaths annually could be directly attributable to AMR. Regions with large populations and weaker healthcare regulations, particularly in Asia and Africa, are expected to bear the brunt of this crisis.[9] Rising resistance rates, coupled with the slow pace of new antibiotic development, highlight the urgent need for innovative solutions to combat AMR.

History and Overview of CRISPR-Cas System

Early Discoveries (1987-2002)

The discovery of the CRISPR-Cas system dates back to 1987, when Atsuo Nakata's group in Japan identified a repetitive 24-nucleotide sequence in *Escherichia coli*. These sequences are associated with enzymes responsible for alkaline phosphatase isozyme conversion, marking the first observation of what would later be recognized as a CRISPR sequence. In the same year, Francisco Mojica from the University of Alicante discovered tandem repeats (TREPs) in the archaeon *Haloferax mediterranei* that are conserved across various microorganisms. This observation, coupled with the analysis of 88 species revealed that two-thirds of these organisms had identical "spacer" sequences, suggesting a role in adaptive immunity in prokaryotes.[17] In 2002, Makarova et al coined the term "Clustered Regularly Interspaced Short Palindromic Repeats" (CRISPRs) and identified genes adjacent to CRISPR regions involved in genome repair, which were later named CRISPR-associated (Cas) genes. This discovery was pivotal in linking CRISPR sequences with the functional immune system of bacteria.[18]

Advancing Understanding (2005 - 2011)

In 2005, Mojica et al discovered that CRISPR loci contained sequences matching a DNA prophage in *Yersinia pestis*.[19] This further supports the role of CRISPR-Cas systems in bacterial immunity.[19] In 2006, Makarova et al conducted a computational analysis that furthered our understanding of the relationship between CRISPR and Cas genes in prokaryotic immune systems.[20] This work laid the foundation for subsequent research on the evolutionary connections between CRISPR systems and Cas proteins.[21] Barrangou et al in 2007 demonstrated that CRISPR-Cas systems provide resistance against viral infections in prokaryotes, confirming the system's role as an adaptive immune mechanism.[22] This was further reinforced in 2011 when Sapranauskas et al successfully transferred the CRISPR-Cas system from *Streptococcus thermophilus* to *E. coli*, establishing the concept of CRISPR as a "DNA memory bank" for microorganisms.[23]

CRISPR-Cas Systems in Genome Editing (2013-Present)

By early 2013, CRISPR tools were effective in making targeted changes to the mammalian genome, marking the beginning of a new era in gene therapy. Since then, CRISPR technology has rapidly evolved, with successful repair of mutations in tissue and animal models of monogenic diseases such as Duchenne muscular dystrophy (DMD), ornithine transcarbamylase (OTC) deficiency, and cataracts.[24] The CRISPR-Cas system is primarily acquired through horizontal gene transfer, which includes transformation, conjugation, and transduction. Bacteria and archaea can acquire CRISPR sequences from other microorganisms, which are then integrated into their genomes to defend against foreign genetic material, such as viruses.[25] Notably, this adaptive immune system is present in approximately 87% of archaeal genomes and 50% of bacterial genomes.[26]

Functional Mechanisms and Types of CRISPR-Cas Systems

CRISPR-Cas systems are composed of short repetitive DNA sequences (CRISPR) separated by spacer regions corresponding to viral or plasmid DNA sequences.[27] These systems operate in three primary phases: adaptation, expression, and interference. In the adaptation phase, bacteria incorporate a 30-base pair DNA fragment from foreign DNA into the CRISPR locus, a process facilitated by Cas1, Cas2, and Cas4 proteins, with the new spacer recognized by the Protospacer Adjacent Motif (PAM). During the expression phase, DNA is transcribed into pre-crRNA, which forms a hairpin structure via palindromic repeats. Cas6 protein cleaves the 5′ end to produce mature crRNA. In the interference phase, crRNA and Cas proteins form a complex that targets and cleaves the invading DNA, providing immunity against future

infections.[28] The bacterial machinery processes a single chimeric guide RNA (sgRNA) to produce mature gRNA, combining CRISPR RNA (crRNA) and transactivating crRNA (tracrRNA). CRISPR-Cas loci include the CRISPR array with spacers and short repeated sequences (repeats), whereas protospacers serve as spacers in these arrays.[29]

CRISPR-Cas systems are divided into two classes and six types, with distinct mechanisms. Class 1 includes Types I, III, and IV, which use multiprotein complexes for interference functions (Figure 1). For example, type I systems use a crRNA and Cas3 complex to cleave DNA during interference.[30–32] Class 2 comprises Types II, V, and VI, which are characterized by single-protein effectors such as Cas9 in Type II systems (Figure 2). Cas9 is widely used in genome editing owing to its simplicity.[33] Recent studies have identified diverse Type V and VI systems with unique mechanisms involving tracrRNA and Cas12 or Cas13 proteins, respectively, targeting DNA and RNA (Figure 1).[34,35] These findings underscore the complexity and adaptability of bacterial immune mechanisms, which are now leveraged for innovative applications in genetic engineering and biotechnology.[35,36] Advances in CRISPR technology have focused on the development of antimicrobial delivery methods, such as bacteriophage particles, nanoparticles, and conjugation techniques (Table 2). Bacteriophage delivery involves the insertion of CRISPR DNA into phage genomes or equipping phagemids with CRISPR components to target bacterial pathogens. Studies have demonstrated the successful integration of Type I CRISPR-Cas systems into λ phage genomes, enhancing their therapeutic efficacy against *Clostridium difficile*.[33,36] Nanoparticles (1–100 nm) are promising for delivering CRISPR antimicrobials by protecting nucleic acids and aiding targeted delivery, although their safety and packaging efficiency are challenging.[37–39] Conjugation, although less studied, can transfer CRISPR components to Gram-negative and Gram-positive bacteria through plasmids, potentially addressing multidrug-resistant pathogens.[40,41]

Potentials of CRISPR Technology in Combating Antimicrobial Resistance

The CRISPR-Cas system noted for its precision and adaptability in genome editing shows significant potential against AMR. Its ability to specifically target and eliminate pathogens makes it ideal for the development of new antimicrobial strategies. This technology operates through spacer sequences, PAM sequences, and Cas proteins, enabling precise interference at specific chromosomal sites. However, further bioinformatics analysis is necessary to optimize these methods.[65–67] Recent advances have highlighted various innovative applications of CRISPR-Cas to address AMR. For instance, Wang et al created a "scissors plasmid" using a temperature-sensitive shuttle plasmid with a guide RNA (gRNA) to direct Cas9 to specific DNA sequences, facilitating precise DNA editing essential for targeted antimicrobial interventions.[51] Bikard et al used the Cas9 nuclease from type II CRISPR systems with a 20-nucleotide guide RNA (crRNA) to selectively kill specific bacterial species, demonstrating selective targeting of antibiotic-resistant bacteria

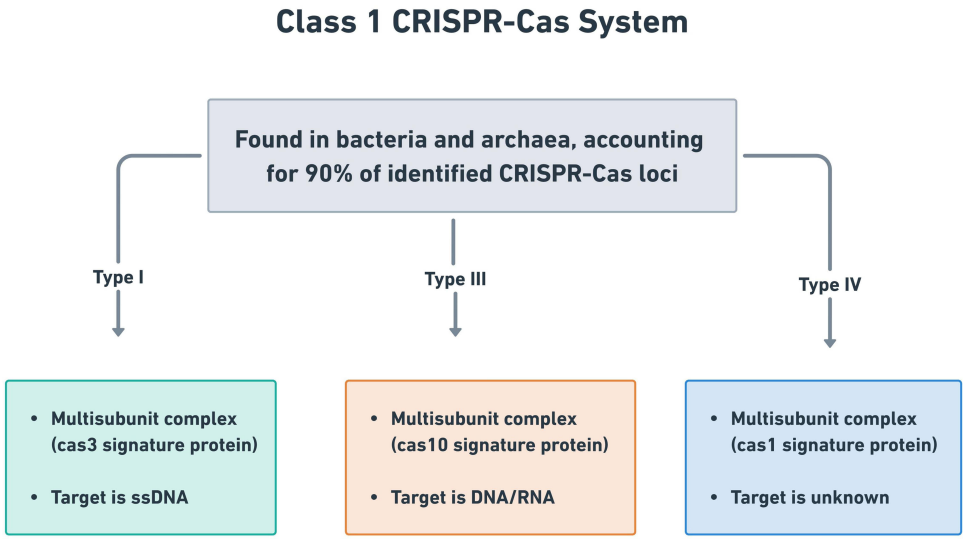

Figure 1 Class 1 CRISPR-Cas Systems: Distribution and Types.

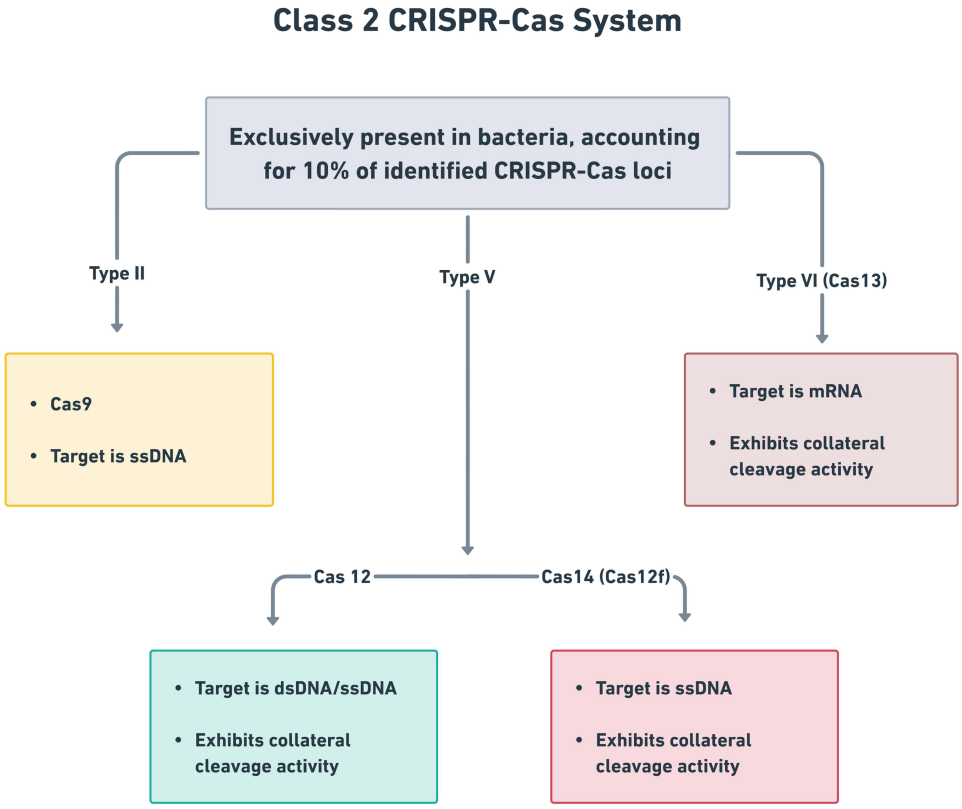

Figure 2 Class 2 CRISPR-Cas Systems: Distribution and Types.

while sparing non-target bacteria, achieved by using bacteriophages to deliver the CRISPR Type II system.[17,68] Liu et al introduced the PRESA (Phage-Delivered Resistance Eradication with Subsequent Antibiotic) treatment, which combined CRISPR-Cas9 with low-cost antibiotics. This approach blocked the transfer of antibiotic-resistant plasmids and

Table 2 Current CRISPR-Cas-Based Antimicrobials in Development*

CRISPR-Cas system (type)	Pathogen	Delivery system	Target location	Gene target	Reference
In Vivo Study					
CRISPR-Cas9 (Type II)	*E. coli*	Cri-nanocomplex (carbon quantum dots)	Chromosome (*pap* gene cluster)	*papG*	[42]
	E. coli	Conjugative plasmid (TP114 plasmid)	Chromosome (AMR cassette inserted in *glmS* terminator)	*Cat*	[43,44]
	E. faecalis	Conjugative plasmid (PRP pPD1)	Plasmid (pAM771, pCF10)	*ermB*	[45]
				tetM	
	E. faecalis	Conjugative plasmid (PRP pPD1)	Plasmid (pAM714)	*repB*	[46,47]
	S. aureus	Bacteriophage (temperate phage)	Chromosome (thermonuclease encoding region)	*Nuc*	[48,49]
	S. aureus	MGEs (staphylococcal pathogenicity islands [SaPIs])	Chromosome (*agr* and listeriolysin encoding regions)	*agrA*	[50]
				Hly	
	S. aureus	Bacteriophage (phiNM1 phage)	Chromosome (kanamycin resistance encoding regions)	*Aph-3*	[51]
				mecA	

(*Continued*)

Table 2 (Continued).

CRISPR-Cas system (type)	Pathogen	Delivery system	Target location	Gene target	Reference
CRISPR-Cas3 (Type I)	*C. difficile*	Bacteriophage (temperate phage)	Chromosome (CR11 array)	ND	[52]
In Vitro Study					
CRISPRi (derived from CRISPR-Cas9)	*E. coli*	Conjugative plasmid (recombinant plasmids)	AcrAB-TolC-associated mRNAs	*acrA, acrB*	[53]
				tolC	
	M. abscessus	Conjugative plasmid (pLJR962)	Peptidoglycan biosynthesis-associated mRNAs	*pbpB*	[54]
				cwlM	
CRISPR-Cas9 (Type II)	*E. coli*	Conjugative plasmid (pSC101)	Plasmid (pNDM-5)	*blaNDM-5*	[55]
	E. coli	Conjugative plasmid (suicide plasmid)	Plasmid (multiple AMR-bearing plasmids)	*mcr-1*	[56]
				blaKPC-2	
				blaNDM-5	
	E. coli	Conjugative plasmid (transposon-associated suicide plasmid)	Chromosome and plasmid (colistin resistance encoding regions)	*mcr-1*	[57]
	Enterobacteriaceae spp.	Conjugative plasmid (pSB1C3)	Plasmid (pSB1A2)	*blaTEM-1*	[58]
	E. coli	MGEs (pro-active genetic system)	Plasmid (*bla* harboring pET)	*Bla*	[59]
	E. faecium	Conjugative plasmid (pVDM1001)	Chromosome (*lac* operon encoding region)	*lacL*	[60]
	S. aureus	Nonconjugative plasmid (Apa I-cut pLI50)	Chromosome (WTA biosynthesis encoding regions)	*tarO*	[61]
				tarH	
				tarG	
	S. aureus	Cri-nanocomplex (polymer-derivatized SpCAS9)	Chromosome (PBP2a encoding regions)	*mecA*	[62]
CRISPR-Cas3 (Type I)	*E. coli*	Bacteriophage (λ phage, T7 phage)	Plasmid (pNDM, pCTX)	*ndm-1*	[63]
				ctx-M-15	
CRISPR-Cas13a (Type IV)	*E. coli*	Phagemids (Cas13a encapsulated with bacteriophage capsid)	Chromosome and plasmid (multiple AMR-encoding regions)	*blaIMP-1*	[64]
				blaOXA-48	
				blaVIM-2	
				blaNDM-1	
				blaKPC-2	
				mcr-1 mcr-2	

Abbreviations: *AMR, antimicrobial resistance; Cri-nanocomplex, nanosized CRISPR complex; MGE, mobile genetic element; ND, nondisclosed; PBP2a.

prevented the emergence of resistant mutants, thereby restoring the efficacy of inexpensive antibiotics.[17,69] Similarly, Wang et al developed the ATTACK strategy, which integrates CRISPR-Cas systems with toxin-antitoxin modules to target and kill multidrug-resistant pathogens.[70] The use of innovative delivery methods has further enhanced the application of CRISPR systems to combat AMR. Bikard et al utilized bacteriophages to deliver the CRISPR Type II system via a phagemid vector, achieving selective killing of antibiotic-resistant bacteria.[16] Additionally, Citorik et al

employed *E. coli* and its filamentous phage M13 to target antibiotic-resistant plasmids, demonstrating variable efficiency across different infection models and potentially improving survival rates in specific models.[68,71]

Despite these notable advancements, the use of the CRISPR-Cas system remains challenging. High-fidelity Cas9 nucleases possess genome-wide off-target effects, necessitating further refinement to enhance their specificity.[72] The effectiveness of the technology varies among bacterial species owing to differences in evolutionary history and CRISPR locus formation. For example, enterococci show a clear negative association with antibiotic resistance, whereas *E. coli* exhibits less of an association.[73,74] Moreover, the CRISPR-Cas system can prevent horizontal gene transfer in some bacteria, such as *S. epidermidis*, but its impact on plasmid epidemiology in *E. coli* is unclear.[68,72] CRISPR-Cas systems are being investigated for innovative therapeutic uses beyond gene editing, such as effectively editing fungi such as *Candida albicans*, Aspergillus, and Cryptococcus, which could enhance treatment outcomes and reduce environmental impacts.[75,76] In diagnostics, combining CRISPR-Cas9 with nucleic acid sequence-based amplification (NASBA) enhances the differentiation of closely related viral strains, making tests more responsive and adaptable.[77] Furthermore, CRISPR-Cas9 is increasingly being used for epigenetic modifications (Table 2), such as targeted DNA methylation, to induce gene expression and improve cell therapy outcomes.[72,78]

Neutralizing Effect of CRISPR Technology on Antimicrobial Resistance

The CRISPR-Cas system uses an RNA-guided nuclease mechanism to precisely target and cut specific DNA sequences to address AMR. By directing Cas proteins to cleave DNA sequences with matching protospacers, the system can target resistance genes.[51] However, recent research has indicated these risks. Targeting bacterial DNA using CRISPR-Cas can induce toxic effects, causing irreversible genetic damage and cell death. This highlights the need for effective delivery methods for RNA-guided nucleases to combat resistant pathogens.[79] Advances in CRISPR-Cas delivery include polymer-coated CRISPR nanocomplexes, plasmid-carrying bacteria, and bacteriophages, which show promise in controlling antibiotic-resistance genes in bacterial populations.[68] For instance, Gomma et al used the Type I-E CRISPR-Cas system in *E. coli*, targeting multiple critical genome sequences, such as ftsA, nusB, msbA, and asd, demonstrating that targeting multiple sites was as effective as targeting single locations. Despite having a potent delivery method, they used transformation, focusing on chromosomal genes unique to some populations rather than resistance genes on extrachromosomal elements.[80] Bikard et al used a Cas9 phagemid to eliminate methicillin-resistant Staphylococcus aureus (MRSA) strains from a mixed bacterial population. They engineered a CRISPR-Cas9 plasmid targeting tetracycline-resistant plasmids, which significantly reduced tetracycline resistance. After treatment, *S. aureus* USA300Φ decreased from 50% to 0.4%, with similar efficiency observed when targeting the enterotoxin sek gene.[81] CRISPR-Cas9 technology has been effective in eliminating carbapenem-resistant plasmids and restoring their susceptibility to carbapenems.[82,83] Similar findings have been reported for *E. coli* and *Klebsiella pneumoniae*.[82,83] Wan et al reported that CRISPR-Cas9 could effectively target and eliminate colistin resistance genes in MCR-1 plasmids.[79] CRISPR-Cas systems also show promise for gram-positive bacteria, which are implicated in the etiology of the most severe infections. Studies on *Enterococcus faecalis*, a common hospital-acquired infection, have revealed that CRISPR systems are crucial in preventing the acquisition of resistance genes. Variations in CRISPR loci influence the effectiveness of targeting resistance genes, and CRISPR-Cas9 has been shown to reduce resistance in *E. faecalis* by targeting genes such as tetM and ermB.[84,85] However, challenges remain, particularly the development of effective CRISPR delivery systems. Current research focuses on using phages, conjugative systems, and polymeric nanoparticles to improve CRISPR delivery and address dysbiosis and microbial community manipulation.[86] Although CRISPR-based nucleases show potential as specific antimicrobials, reliable delivery systems are essential for broader application and effectiveness.[72,87]

Ethical, Social, and Regulatory Implications of CRISPR Technology

Since its introduction in 2012, CRISPR technology has revolutionized molecular biology, particularly in addressing AMR through precise bacterial genome modifications. The unprecedented ability of this technology to modify bacterial genomes has sparked significant ethical debates within the scientific community, especially regarding its implications for microbial ecosystems and human health.[88,89] Several critical ethical concerns have emerged, specifically related to AMR applications. The potential for horizontal gene transfer between modified and unmodified bacteria raises concerns

regarding the uncontrolled spread of engineered genetic elements in microbial populations.[90] The environmental implications of CRISPR-mediated changes in bacterial populations highlight the need for robust regulation to manage the risks associated with the release of engineered microorganisms into ecosystems.[89,91] The widespread use of CRISPR-based antimicrobials requires careful consideration of their impact on beneficial microbiota and the potential emergence of new resistance mechanisms.[92,93] The potential for off-target mutations in non-pathogenic bacteria could lead to unexpected ecological consequences and create new health challenges.[94,95]

The use of CRISPR technology to address AMR raises questions about equitable access to treatment, particularly in developing countries where the burden of resistant infections is the highest.[96,97] Regulatory frameworks must balance the urgent need for new antimicrobial strategies with careful oversight of genetic modifications in microorganisms.[98] The technical viability of employing CRISPR for AMR control faces several challenges, particularly the complexity of resistance mechanisms and their interactions with environmental factors.[89,91,94] These concerns have led to calls for establishing international standards for the development and deployment of CRISPR-based antimicrobials, ensuring both safety and efficacy while preserving beneficial microbial diversity.[99]

Limitations of CRISPR in Antimicrobial Applications

Off-Target Effects (OTEs)

A major challenge with CRISPR/Cas9 is the occurrence of off-target effects (Table 3), which can occur at frequencies ≥50%. These effects are particularly concerning when targeting resistance genes, as unintended modifications could potentially create new resistance mechanisms or affect beneficial bacteria.[100] Researchers have developed various strategies to mitigate these effects, including engineered Cas9 variants designed to reduce the number of OTEs while maintaining editing efficiency. For example, the SpCas9-HF1 variant demonstrated reduced off-target activity compared to wild-type SpCas9 when targeting resistance genes.[101] These innovations are crucial for ensuring the precise targeting of resistance genes while preserving beneficial bacterial populations (Table 3).[102]

Impact on the Gut Microbiome

CRISPR-Cas9 technology, which is promising for targeting antibiotic-resistant bacteria, poses potential risks to beneficial microbiota. This application can inadvertently disrupt essential bacterial communities, potentially creating ecological niches for opportunistic pathogens or new resistant strains [103]. This disruption can lead to secondary infections or the emergence of new resistance patterns. Maintaining microbial diversity while targeting specific resistant bacteria remains a significant challenge for CRISPR-based antimicrobial strategies.

Phage Delivery Challenges

The delivery of CRISPR systems via bacteriophages presents both opportunities and challenges for targeting antibiotic-resistant bacteria. Bacteriophages or phages, are viruses that specifically infect bacteria and can be engineered to deliver CRISPR components to bacterial cells.[5] However, the use of phages as delivery vehicles is limited by their narrow host range, which means that each phage typically targets only a specific bacterial species or strain. This limitation reduces the versatility of phage-based CRISPR delivery systems (Table 3).[104] This specificity is particularly problematic when dealing with mixed infections involving multiple drug-resistant pathogens. Moreover, bacteria can evolve resistance mechanisms against phages, such as altering their surface receptors to prevent phage attachment or acquiring anti-phage defense systems, such as anti-CRISPR proteins. These evolutionary adaptations can compromise the effectiveness of phage-based CRISPR delivery systems, potentially leading to treatment failure in antibiotic-resistant infections.[105]

Delivery and Treatment Efficacy

The effectiveness of CRISPR-based antimicrobial treatments is heavily dependent on the efficient and accurate delivery of CRISPR components to resistant bacterial populations.[106] However, this process faces significant challenges, as bacterial DNA repair mechanisms can introduce unintended modifications, such as deletions or duplications, potentially creating new resistant phenotypes or compromising treatment efficacy.[107] To address these issues in targeting resistant

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Table 3 Comparison of CRISPR/Cas Protein Properties

Characteristics	CRISPR/Cas9	CRISPR/Cas12	CRISPR/Cas13	CRISPR/Cas14
DNA catalytic domain	RuvC, HNH	RuvC-like nuclease domain, Nuc-domain	HEPN domains	RuvC
Target	Double-stranded DNA	Double-stranded DNA	Single-stranded RNA	Single-stranded DNA
Collateral activity	No	Yes	Yes	Yes
DNA recognition	sgRNA (crRNA in complex with tracrRNA)	crRNA	crRNA	crRNA and tracrRNA
PAM requirements	NGG, NAG for SpCas9, and other PAM variants for Cas9 orthologs	TTTN, TTTV(V = G, C, or A)For AsCpf1 from *Acidaminococcus* or LbCpf1 from *Lachnospiraceae*, and other PAM variants for Cas12 orthologs	Requires protospacer flanking sequence–A, U, or C	None
Specificity	Regular SpCas9 tolerates mismatches, but high-fidelity variants exist	Cas12a has been successfully used for gene editing in vivo without any deleterious off-target effects	RNA-editor, no damage to DNA may occur	Cleaves ssDNA with high fidelity-sensitive to even a single mismatch in the target sequence
Ease of delivery	Easily delivered using multiple techniques	Easily delivered using multiple techniques	Easily delivered using multiple techniques	Easily delivered using multiple techniques
Limitations	GC-rich DNA targetsPossible off-target effects (especially for SpCas9)	AT-rich DNA targets	Needs to be constitutively expressed to maintain the editing effect	Targets ssDNA
Multiplexing	Easy	Easy	Easy	Easy
Ongoing clinical trials	Yes 57 studies https://clinicaltrials.gov/search?intr=CRISPR%2FCas9andpage=6	Yes 2 studies https://clinicaltrials.gov/search?intr=CRISPR%20Cas12a	Yes 1 study https://clinicaltrials.gov/search?intr=CRISPR%20Cas13	No
Use in academic laboratories	30,209 results in PubMed® (https://pubmed.ncbi.nlm.nih.gov/, accessed on 8 August 2024 with search term "CRISPR Cas9"	2130 results in PubMed® (https://pubmed.ncbi.nlm.nih.gov/, accessed on 8 August 2024 with search term "CRISPR Cas12a or Cpf1"	439 results in PubMed® (https://pubmed.ncbi.nlm.nih.gov/, accessed on 8 August 2024 with search term "CRISPR Cas13"	32 results in PubMed® (https://pubmed.ncbi.nlm.nih.gov/, accessed on 8 August 2024 with search term "CRISPR Cas14"
First time mentioned	2011	2017	2017	2018

bacteria, researchers employ selection procedures to minimize unwanted mutations; however, these methods are not foolproof when dealing with diverse bacterial populations.[108] The presence of genetically diverse bacterial populations within infection sites can hinder the precise targeting of resistance genes and complicate the elimination of resistant strains. This heterogeneity presents a significant obstacle to achieving uniform genetic modifications across bacterial populations, particularly in clinical settings with mixed infections.[72]

Cell Death and Safety Concerns

CRISPR-induced double-strand breaks in bacterial DNA, which are effective in targeting antibiotic resistance genes, can lead to unintended consequences in microbial populations. This occurs when DNA breaks trigger cellular repair mechanisms that can potentially create new resistance phenotypes or affect nontarget bacteria.[109] In antibiotic-resistant bacteria, these DNA breaks can lead to the successful elimination of resistance genes or, in some cases, trigger survival mechanisms that might result in additional mutations.[110] This dual-edge effect highlights the safety concerns associated with CRISPR-induced DNA damage in bacterial populations.[111] Reports of large deletions and complex genomic rearrangements in bacteria following CRISPR activity have raised concerns about the potential transfer of modified genetic material to other microorganisms. Alternatives such as non-nuclease dCas9, which can suppress resistance gene expression without causing double-strand breaks, and advancements such as base editors and prime editors offer safer options for targeting antibiotic resistance.[11,112]

Delivery Methods

The delivery of CRISPR components to target antibiotic-resistant bacteria is critical for success and safety. Traditional viral vectors, such as AAVs, are commonly used but can lead to prolonged-expression and increased risk of off-target effects in bacterial populations.[62] Non-viral methods, such as electroporation and microinjection, present challenges when targeting resistant bacteria. While microinjection is mainly suitable for ex vivo applications, electroporation can be toxic because of the high voltage required, potentially affecting both resistant and beneficial bacteria. AAV delivery can lead to potential immunogenicity and off-target effects in bacterial populations.[113] Delivering CRISPR components as ribonucleoprotein (RNP) complexes has shown promise in reducing off-target effects in resistant bacteria owing to their transient presence in cells.[114]

In vivo and ex vivo Applications

Ex vivo CRISPR delivery for targeting antibiotic-resistant bacteria allows for greater safety and technical feasibility but is limited to bacterial populations that can be isolated and cultured from infection sites.[45] In vivo applications for treating resistant infections, including systemic and local administration, face challenges, such as degradation of CRISPR components by host proteases, clearance by the immune system, and uneven distribution of components at infection sites.[45] The effectiveness of in vivo applications is particularly challenging when targeting biofilm-associated resistant bacteria or deep-seated infections.[115] Despite these challenges, advances in delivery techniques continue to improve the clinical utility of CRISPR in the treatment of antibiotic-resistant infections, particularly through the development of targeted delivery systems.

Immunogenicity

CRISPR technology is associated with immunogenicity concerns when targeting antibiotic-resistant bacteria. Studies have shown that a significant proportion of human subjects possess pre-existing anti-Cas9 antibodies, particularly against commonly used orthologs such as SaCas9 and SpCas9, which could affect the efficacy of CRISPR-based antimicrobial treatments.[116] Although alternative Cas9 orthologs, such as *Campylobacter jejuni* Cas9 (CjCas9), have shown reduced immunogenicity and robust editing efficiency in animal models, further research is needed to evaluate their safety and efficacy for clinical use.[117] While CRISPR technology holds immense promise, addressing these limitations through continued research and development is essential for enhancing its precision, safety, and effectiveness in therapeutic applications.

Future Prospects for CRISPR in Combating AMR

To enhance the efficacy of CRISPR systems in combating resistant pathogens, researchers have explored a range of novel CRISPR proteins. Cas12a and Cpf1 are advantageous over Cas9 because of their unique structure and high specificity. Clinical trials have highlighted its potential in treating genetic disorders such as sickle cell disease and beta-thalassemia, underscoring its possible application in AMR.[118–120] Cas13, which targets RNA instead of DNA, opens up innovative pathways for managing RNA-based pathogens and modulating gene expression. The discovery of multiple Cas13 variants further expands their applications, allowing for precise targeting and interference with pathogenic RNA.[121–124] Similarly, Cas14, a miniature CRISPR protein, provides unique advantages owing to its ability to cleave single-stranded DNA without a PAM sequence, offering flexibility and efficiency in genome editing.[125–127] Advanced screening techniques and omics technologies are pivotal to enhancing our understanding of AMR. Genome-wide knockout screens using CRISPR-guided RNA libraries enable the systematic identification of genes involved in AMR pathways.[128] The integration of these screens with single-cell omics methodologies, such as PERTURB-seq and CRISP-seq, allows for detailed tracking of gene expression changes and genetic interactions. Lineage tracing and molecular recording techniques further contribute by monitoring cell proliferation and reconstructing cell lineage trees, thereby providing insights into AMR evolution.[129,130]

CRISPR,s therapeutic potential of CRISPR is evident in both ex vivo and in vivo applications. Ex vivo CRISPR editing of patient-derived cells is a powerful strategy for treating genetic disorders, such as hemoglobinopathies and cancers, and has shown promise for dystrophin deficiencies and retinal dystrophies.[129–132] In vivo CRISPR editing through localized or systemic delivery highlights the potential to address resistant pathogens, necessitating advancements in the delivery systems. Ultracompact RNA-guided nucleases, such as Cas12f, are being applied in developing targeted antimicrobials or modifying pathogens to improve susceptibility to treatments.[132] DNA polymerase-based editing technologies, such as click editing, enable precise genetic alterations and potentially reduce bacterial resistance.[133,134] CRISPR-guided recombinases and transposons facilitate large DNA insertions and efficient gene sequence introduction, even in post-mitotic cells.[132] Retrotransposons and epigenome editing offer strategies for integrating long DNA sequences and modulating gene expression without altering the DNA sequence.[129,135,136] Advancements in delivery systems such as lipid nanoparticles, cell-penetrating peptides, and engineered viral particles are crucial for improving the efficiency and specificity of CRISPR-based antimicrobial strategies[137,138] In addition, artificial intelligence and deep learning improve genome editing precision by predicting effective targets and reducing unintended effects, thereby enhancing AMR-specific intervention development.

Conclusion

CRISPR-Cas systems have advanced from early technologies that utilize endogenous repair mechanisms and site-specific DSBs to sophisticated tools that target AMR. Recent innovations, such as base editing and prime editing, have provided safer and more predictable outcomes by enabling direct DNA modifications without DSBs. Future developments are expected to further refine these technologies, emphasizing the precise insertion of large gene segments and enhanced gene regulation through epigenome editing. Key areas for future research include improving the specificity, reducing off-target effects, and optimizing delivery methods. This includes developing less immunogenic vectors and addressing the challenges in targeting non-blood organs and specific cell types. Artificial intelligence has been poised to enhance CRISPR technology by improving genomic landscape modelling, predicting outcomes, and designing more effective tools. Although first-generation CRISPR technologies are already in clinical use, overcoming challenges in delivery and minimizing unintended long-term effects remain critical.

Abbreviations

AMR, Antimicrobial Resistance; WHO, World Health Organization; CDC, Centers for Disease Control and Prevention; EARS-Net, European Antimicrobial Resistance Surveillance Network; GLASS, Global Antimicrobial Surveillance System; MRSA, Methicillin-resistant Staphylococcus aureus; GDP, Gross Domestic Product; OECD, Organisation for Economic Co-operation and Development; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; Cas, CRISPR-associated; DMD, Duchenne Muscular Dystrophy; OTC, Ornithine Transcarbamylase; PAM, Protospacer

Adjacent Motif; crRNA, CRISPR RNA; tracrRNA, Transactivating CRISPR RNA; sgRNA, Single Chimeric Guide RNA; gRNA, Guide RNA; PRESA, Phage-Delivered Resistance Eradication with Subsequent Antibiotic; NASBA, Nucleic Acid Sequence-Based Amplification; DSBs, Double-Strand Breaks; OTEs, Off-Target Effects; HSPCs, Hematopoietic Stem and Progenitor Cells; AAVs, Adeno-Associated Viruses; dCas9, Dead Cas9 (a modified form of Cas9 that does not cut DNA); Cas12a, CRISPR Associated Protein 12a (also referred to as Cpf1); Cas13, CRISPR Associated Protein 13; Cas14, CRISPR Associated Protein 14; RNA, Ribonucleic Acid; DNA, Deoxyribonucleic Acid; CRISPR-Cas, Referring to the CRISPR system that includes both the CRISPR array and the associated Cas proteins; PERTURB-seq, Perturbation and sequencing (single-cell sequencing technology); CRISP-seq, CRISPR-based sequencing; Ex vivo, Outside the living organism; In vivo, Within the living organism; AI, Artificial Intelligence.

Data Sharing Statement

Not applicable because no new data or databases were used in this study.

Ethical Approval

Approval from the Ethics Committee was not required.

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