

# The Application of the CRISPR-Cas System in Antibiotic Resistance

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**Abstract:** The emergence and global epidemic of antimicrobial resistance (AMR) poses a serious threat to global public health in recent years. AMR genes are shared between bacterial pathogens mainly via horizontal gene transfer (HGT) on mobile genetic elements (MGEs), thereby accelerating the spread of antimicrobial resistance (AMR) and increasing the burden of drug resistance. There is an urgent need to develop new strategies to control bacterial infections and the spread of antimicrobial resistance. The clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated proteins (Cas) are an RNA-guided adaptive immune system in prokaryotes that recognizes and defends against invasive genetic elements such as phages and plasmids. Because of its specifically target and cleave DNA sequences encoding antibiotic resistance genes, CRISPR/Cas system has been developed into a new gene-editing tool for the prevention and control of bacterial drug resistance. CRISPR-Cas plays a potentially important role in controlling horizontal gene transfer and limiting the spread of antibiotic resistance. In this review, we will introduce the structure and working mechanism of CRISPR-Cas systems, followed by delivery strategies, and then focus on the relationship between antimicrobial resistance and CRISPR-Cas. Moreover, the challenges and prospects of this research field are discussed, thereby providing a reference for the prevention and control of the spread of antibiotic resistance.

**Keywords:** antibiotic resistance, CRISPR-Cas, horizontal gene transfer

## Introduction

In recent years, antibiotic resistance has intensified due to the extensive use of antibiotics.<sup>1</sup> Antimicrobial resistance genes (ARGs) may be carried on the bacterial chromosome, and mobile genetic elements (MGEs) such as plasmid, and transposons. The resistance mechanisms mainly include the production of inactivated enzymes and modified enzymes ( $\beta$ -lactamases, aminoglycoside modifying enzymes), changes in the target site of the drug, the alteration of membrane permeability, increased active efflux pump system expression, formation of the bacterial biofilm (BBF).<sup>2,3</sup> BBF (adaptive resistance) serves as a diffusion barrier to limit the access of antibiotics to the bacterial cells.<sup>4</sup> Loss of efficacy in antibiotics due to antibiotic resistance in bacteria is an urgent threat to the success of microbial infection therapy. The spread of antibiotic-resistant bacteria poses a substantial threat to morbidity and mortality worldwide.<sup>5,6</sup> ARGs can spread through vertically inherited chromosomal mutations and horizontal gene transfer (HGT) mediated by MGEs among bacteria.<sup>7</sup> The acquisition of ARGs mediated by HGT is the main reason for the spread of drug resistance.<sup>8</sup> HGT is mainly driven by conjugation, transduction, and natural transformation.<sup>9</sup> MGEs share their genetic elements carrying resistance genes with other non-resistant bacterial species via HGT, which promoted the accumulation and dissemination of ARGs in Gram-negative and Gram-positive bacteria.<sup>10,11</sup> To treat bacterial infections and prevent and control the spread of antibiotic resistance, the research and development of new agents with therapeutic potential have attracted more attention worldwide.

Bacteria have evolved multiple defense mechanisms under long-term selection pressure, such as restriction-modification systems and CRISPR-Cas system, which act as innate immune and adaptive systems in bacteria, and

significantly affect the spread of antibiotic resistance genes and phage infection.<sup>12–14</sup> CRISPR-Cas systems are considered as barriers to HGT in bacteria.<sup>15</sup> CRISPR-Cas system can be used to re-sensitize drug-resistant bacteria to antibiotics by specifically eliminating the plasmids carrying antibiotic resistance genes.<sup>16</sup> CRISPR has been highlighted as an important tool to prevent and control the spread of bacterial resistance.

In this review, we describe the structure and working mechanism of the CRISPR-Cas system, introduce the delivery method, elucidate the relationship between antimicrobial resistance and CRISPR-Cas, discuss the status of clinical challenges, which aims at providing new ideas for the prevention and control of bacterial resistance. Overall, CRISPR-Cas systems have different effects on antibiotic resistance in different bacteria. The CRISPR-Cas system plays an important role in limiting the spread of antibiotic resistance.

## The Structure and Working Mechanism of the CRISPR-Cas System

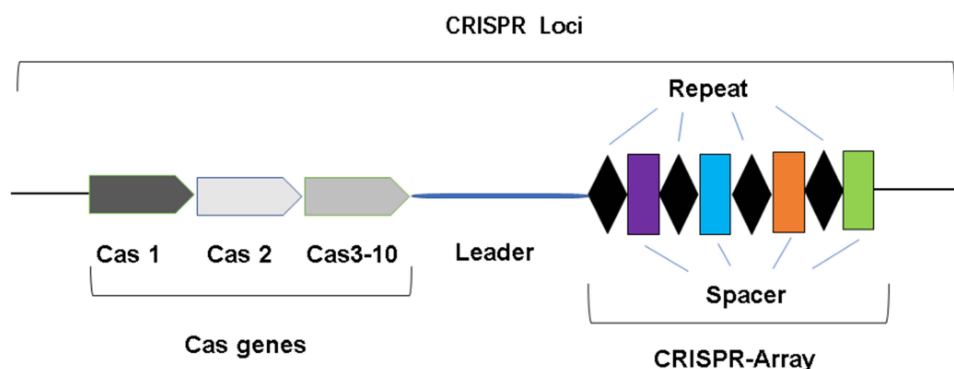
The CRISPR system is composed of a CRISPR array and a set of CRISPR-associated (Cas) genes.<sup>17</sup> The CRISPR array consists of short repeat sequences separated by spacer sequences.<sup>18</sup> The CRISPR array is typically preceded by an A-T-rich leader sequence containing a promoter used to initiate transcription of the repeat and spacer sequences<sup>19,20</sup> (see Figure 1).

The CRISPR-Cas system is mainly divided into two categories according to its constituent proteins and modes of action.<sup>21,22</sup> Class 1 utilizes multi-protein effector complexes to degrade nucleic acids, and can be further divided into type I, type III, and type IV; Class 2 uses single-protein effector complexes to degrade nucleic acids, which can be further divided into type II, type V and type VI.<sup>23,24</sup> Types II CRISPR-Cas systems are more widely studied and applied to eliminate resistance genes due to their relatively simple structure.<sup>25</sup> In recent years, Type I CRISPR-Cas systems have also been developed as genetic manipulation tools to eliminate resistance genes.<sup>26</sup>

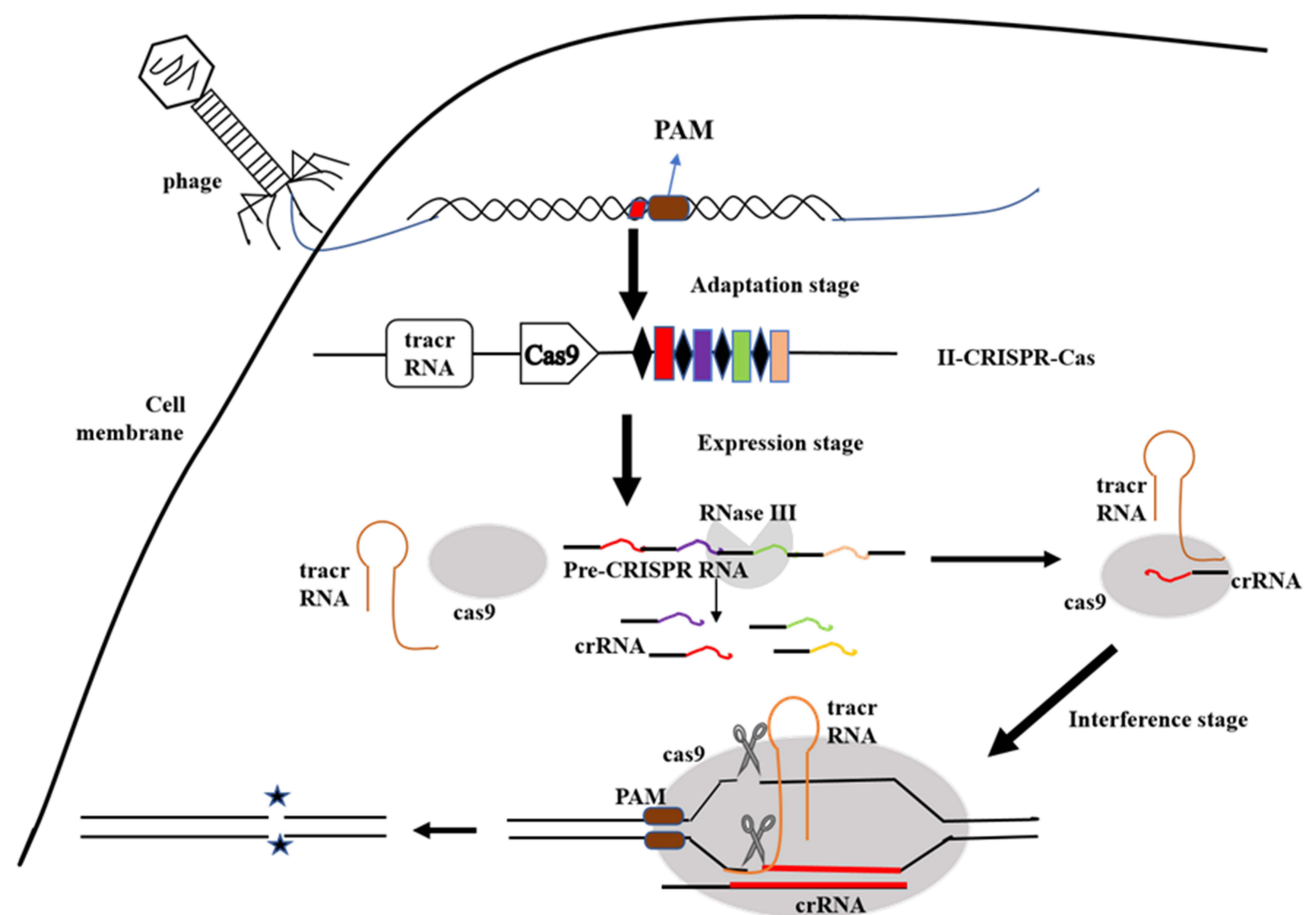
The bacterial defense mechanism of the CRISPR/Cas systems includes three stages.<sup>14,27</sup> (i) Adaptation stage: acquisition of spacer sequences.<sup>28,29</sup> (ii) Expression stage: generation of the crRNA and Cas protein;<sup>24</sup> (iii) Interference stage: crRNA-guided nucleic acid-targeted cleavage.<sup>30,31</sup> The mature crRNA binds to Cas protein to form a nucleic acid-protein complex, which can recognize sequences complementary to crRNA in nucleic acid and exert endonuclease activity to degrade the nucleic acid near the recognition site<sup>32</sup> (see Figure 2). This specificity allows the CRISPR system to be developed to eliminate specific drug resistance genes, thus controlling horizontal gene transfer and limiting the spread of antibiotic resistance.<sup>33</sup>

## Immune Recognition and Defense Mechanism of I and II CRISPR-Cas System

Type I CRISPR-Cas system is a typical representative of Class 1, and its marker protein is the Cas3 protein containing the phosphohydrolase domain and the helicase domain.<sup>34</sup> Type I CRISPR systems rely on multiple proteins working together in a complex called Cascade to target DNA.<sup>35</sup> After binding to DNA, Cascade recruits the Cas3 protein to cut DNA.<sup>36</sup> The adaptation process of the type I CRISPR-Cas system is mediated by Cas1 and Cas2, which integrate the exogenous DNA segment between its leader sequence and the first repeat of CRISPR to form a new spacer. At the



**Figure 1** The structure of CRISPR system.



**Figure 2** The working mechanism of the CRISPR-Cas system. The bacterial defense mechanism of the CRISPR/Cas systems includes three stages: Adaptation stage: acquisition of spacer sequences; Expression stage: Generation of the crRNA and Cas protein; Interference stage: crRNA-guided nucleic acid-targeted cleavage.

expression stage, the Cas protein processed the pre-CRISPR RNA (pre-crRNA) into mature crRNA and binds into a complex with a group of Cas proteins (cascade), which guides the Cas3 protein to cleavage foreign target sequences.<sup>37</sup>

Class 2 type II CRISPR-Cas9 system has been most widely adapted for immune defense and gene editing.<sup>38</sup> Cas9 contains a unique active site of RuvC at the amino-terminal end and HNH2 in the middle of the protein, playing a role in crRNA maturation and double-stranded DNA shear.<sup>39</sup> At the same time as pre-crRNA is transcribed, trans-activating crRNA (tracrRNA) that has sequence complementarity to CRISPR repeats is also transcribed and stimulates Cas9 and double-stranded RNA-specific RNase III nuclease to process pre-crRNA into mature crRNA.<sup>12,40</sup> After processing, crRNA, tracrRNA, and Cas9 form a complex, which recognizes and binds to the MGE genome.<sup>41,42</sup> Then the complementary protospacer strand is cleaved by the HNH endonuclease domain of Cas9, and the non-complementary strand is cleaved by the RuvC endonuclease domain of Cas9, generating a double-stranded DNA break (DSB) in the invading MGE.<sup>43</sup> The cleavage site of CRISPR/Cas9 is located in the NGG site of the PAM region (Protospacer Adjacent Motif) adjacent to the downstream of the crRNA complementary sequence, and cleaves nucleotides on the nearby target sequence to generate a double-strand break (DSB)<sup>44–46</sup> (See Figure 2).

## Delivery Strategies for CRISPR-Cas Systems

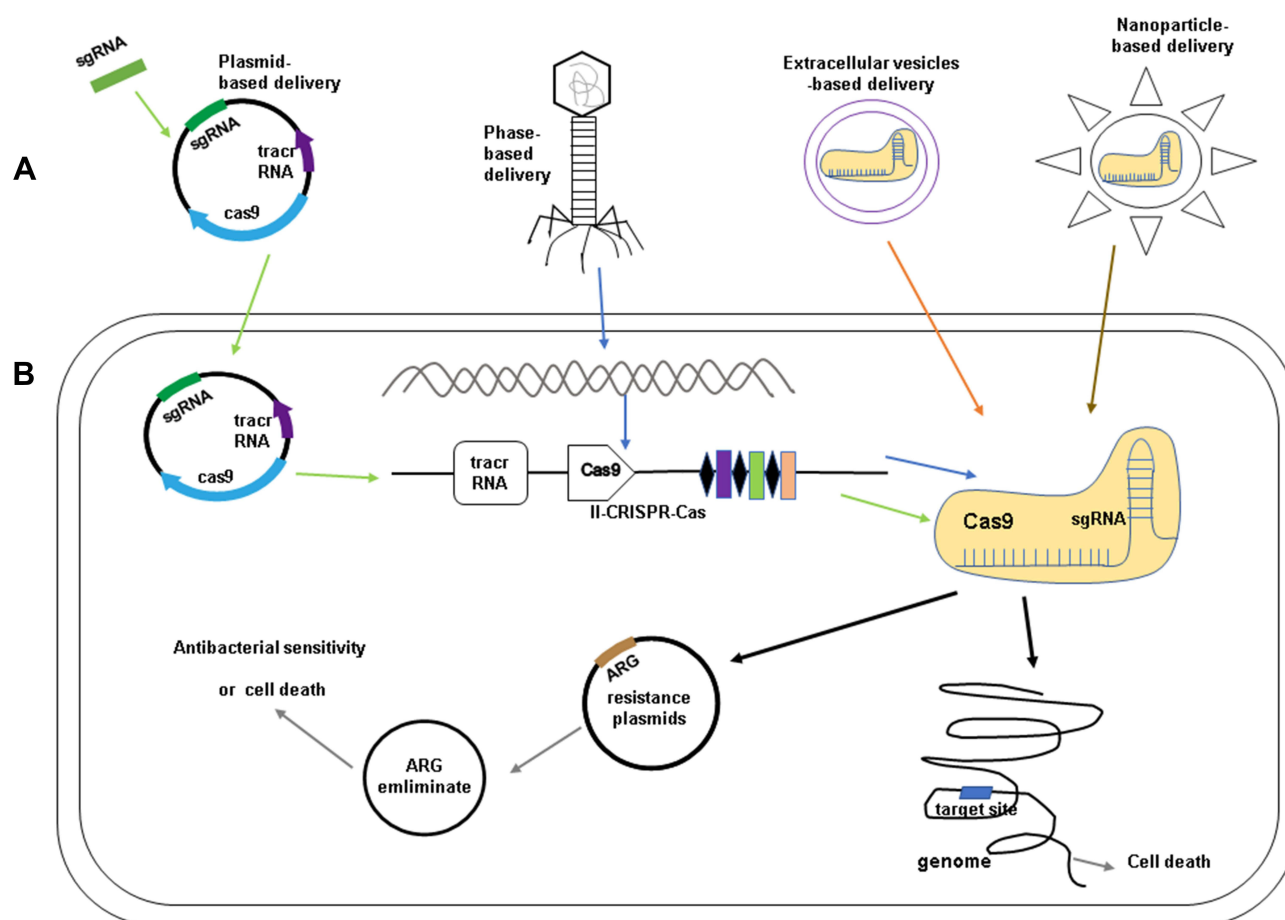
The CRISPR-Cas system has potential applications in the prevention and control of the spread of bacterial resistance caused by drug resistance genes. The direct application of the CRISPR-Cas system is to design the genomic gRNA targeting drug-resistant genes or resistant bacteria to guide the CRISPR-Cas system to cleave the targeted sequences, thereby restoring their antibiotic sensitivity or killing bacteria.<sup>45</sup> The efficient delivery of the CRISPR-Cas system into

microorganisms is the main problem to be solved by using this system to prevent and control bacterial drug resistance.<sup>47</sup> Based on a brief introduction of the working mechanism of the CRISPR-Cas system, we will elucidate the progress of reducing resistance genes and resistance plasmids using the CRISPR-Cas system with plasmids, extracellular vesicles, phage, and nanoparticles as carriers (see Figure 3A).

## Plasmid Vector-Based Delivery of CRISPR-Cas Systems

Integrating the sequences of the CRISPR-Cas system or some of its components into a plasmid vector enables the CRISPR-Cas system to target and cut drug-resistant genes, thereby reducing the resistance of drug-resistant bacteria to antibiotics.<sup>48</sup>

Conjugation is an important mode of bacterial gene transfer that enhances the spread of resistance genes.<sup>49,50</sup> CRISPR-Cas encoding delivery plasmids can reduce the occurrence of antibiotic resistance in *Enterococcal* populations in a sequence-specific manner.<sup>51</sup> Moreover, the introduction of a plasmid vector carrying the pCasCure system into carbapenem-resistant *Enterobacteriaceae* could remove carbapenemase resistance genes such as blaNDM and blaKPC, re-sensitize the resistant bacteria to carbapenems, and have good therapeutic effects on clinical isolation of carbapenem-resistant *Enterobacteriaceae*.<sup>52</sup> The recipient bacteria with the conjugation plasmid can further transmit the CRISPR-Cas system to other recipient bacteria, which greatly expands the application scope of using the CRISPR-Cas system to reduce drug resistance genes.<sup>53</sup> In addition, the targeted antimicrobial plasmids (TAPs) can be used to carry the CRISPR-



**Figure 3** (A) The methods for delivery of CRISPR-Cas components: plasmids-based delivery (Design and synthesize sgRNA targeting the target gene and ligate it into a plasmid vector containing Cas9); phage-based delivery (The CRISPR-Cas system was integrated into the phage genome and delivered with the phage as a vector); Extracellular vesicles (EVs)-based delivery and nanoparticle-based delivery (Cas9 protein and sgRNA form a ribonucleoprotein (RNP) complex and packaged into EVs and nanoparticles). (B) The application of CRISPR-Cas system in antibiotic resistance. When the delivery of CRISPR-Cas systems in the bacteria cells, the antibiotic-resistant genes (ARG) on the plasmids could be eliminated and the bacteria can re-sensitized against antibacterial agents. CRISPR-Cas also shows a strong bactericidal activity and make the cell die after the recognition of the target ARG on plasmid and the target site on genome.

Cas system for efficient transfer to *Escherichia coli* and the highly related Gram-negative *Enterobacteriaceae*, re-sensitize the recipient cells carrying pOXA48 and prevent the spread of drug resistance.<sup>54</sup> Dong et al<sup>55</sup> constructed the conjugative CRISPR/Cas9 system targeting the mobile colistin resistance gene (*mcr-1*) in *Escherichia coli*, this engineered CRISPR/Cas9 system can not only eliminate drug-resistant plasmids and re-sensitize to antibiotics but also make the recipient cell acquire immunity against *mcr-1*. The recombinant plasmid pMBLcas9-sgRNA was able to transfer into clinically isolated *E. coli* carrying different MCR-1 plasmids with a conjugation efficiency of about  $10^{-1}$ , successfully eliminating the multidrug resistance plasmids.<sup>56</sup>

Delivery efficiency is the main factor limiting the clinical application of the CRISPR-Cas system.<sup>57</sup> Improving plasmid conjugation efficiency can expand the application prospect of the CRISPR-Cas system in preventing and controlling drug resistance gene transfer.<sup>58</sup> The pheromone-responsive plasmid (PRP) in *Enterococcus faecalis* has a higher conjugative transfer efficiency than other plasmids.<sup>59</sup> pPD1 plasmid has been used to transfer CRISPR/Cas systems to the Gram-positive *Enterococcus faecalis*. Studies have shown that the pheromone response plasmid (PRP) in *Enterococcus faecalis* synthesizes a protein adhesin under the induction of pheromone secreted by the recipient bacteria without the conjugation plasmid, and promoting the aggregation of the donor and recipient bacteria, thereby increasing the conjugation efficiency of pheromone-responsive plasmids.<sup>60</sup>

## Phage Vector-Based Delivery of CRISPR-Cas Systems

Compared with plasmid vectors, phage vectors not only have a stronger ability to infect host bacteria but also can carry larger DNA fragments, which can be introduced into the CRISPR-Cas system by encoding multiple proteins.<sup>61</sup> In addition, the nucleic acid encapsulated by the phage protein is relatively stable and not easily degraded.<sup>62</sup> The advantage of phage naturally targeting bacteria makes it a preferred delivery tool for researchers.<sup>63</sup>

Using bacteriophage as a vector, the sgRNA and Cas9 designed based on the conserved sequences of  $\beta$ -lactamase mutants were introduced into the target strain, which successfully inactivated more than 200 mutant pathogenic bacteria and re-sensitized to  $\beta$ -lactamase.<sup>64</sup> In addition, a type I CRISPR-Cas3 system containing six Cas genes and multiple spacers targeting *ndm-1* and *ctx-M-15* resistance genes were packaged using lysogenic phages,<sup>65</sup> which could selectively destroy drug-resistant plasmids and restore the susceptibility of host bacteria to multiple antibiotics.<sup>66</sup> Bikard et al<sup>67</sup> used phagemids to carry the CRISPR-Cas9 system to target the resistance gene of *Staphylococcus aureus*, and found that it can destroy the plasmid containing the target gene, and if the target gene is on the chromosome, it will cause bacterial death. Research<sup>68</sup> indicated that a new mild phage packaging CRISPR-Cas9 system can deliver the CRISPR-Cas9 system and target bacterial drug resistance plasmids. CRISPR-Cas9 wrapped by phages can mediate the degradation of DNA of antibiotic resistance genes (ARG) on plasmids but does not cause cell death. However, the CRISPR-Cas13a wrapped by the phage showed a strong bactericidal activity after the recognition of the target ARG on the plasmid.<sup>69</sup>

## Extracellular Vesicles Vector-Based Delivery of CRISPR-Cas Systems

Membrane vesicles (MV) are the lipid membrane nanoparticles first found in Gram-negative bacteria that are also known as outer membrane vesicles (OMV).<sup>70</sup> Bacterial outer membrane vesicles (OMVs) are vesicle-like structures secreted into the extracellular compartment of Gram-negative bacteria during growth.<sup>71,72</sup> OMVs can act as delivery systems for antibiotic resistance genes, virulence genes, or plasmids because these components are protected from DNases when present in the vesicle lumen, thereby facilitating the horizontal gene transfer of DNA.<sup>73,74</sup> Recently, OMVs have been explored as vectors for delivering Cas9 ribonucleoprotein (RNP) for gene editing and gene regulatory purposes.<sup>74</sup> It has been shown that OMVs secreted by *Escherichia coli* can act as carriers for the CRISPR-Cas9 system targeting *Streptococcus agalactiae*, achieving efficient and specific clearance of *Streptococcus agalactiae* in a mixed culture manner.<sup>75</sup> However, the lack of a mechanism to enrich RNP into membrane vesicles limits the efficiency of membrane vesicles as an RNP delivery tool.<sup>74,76</sup>

## Nanoparticle-Based Delivery of CRISPR-Cas

Nanoparticles are small in size and have a strong ability to penetrate biological membranes.<sup>77</sup> After the CRISPR-Cas9 system is encapsulated by nanomaterials, CRISPR-Cas9 is not easily degraded and can be highly efficient and targeted



transported.<sup>78</sup> Many studies have been conducted on the application of nanoparticles as carriers for antibiotics, such as polymeric nanoparticles and nanocrystals, zwitterion amino lipid (ZALs) nanoparticles, exosome-liposome mixed nanoparticles, and cationic lipid nanoparticles, nanocages et al.<sup>79</sup> Kang et al<sup>52</sup> assembled Cas effector proteins and guide RNAs into branched polyethyleneimine nanomaterials, which can be efficiently delivered into methicillin-resistant *Staphylococcus aureus* (MRSA) for more efficient editing of bacterial genomes targeting *mecA*. More studies investigated the role of nanotechnology-based CRISPR-Cas9 delivery systems in controlling and preventing antimicrobial resistance.<sup>80</sup> Although great progress has been made in optimizing the CRISPR-Cas system with nanoparticles for the treatment of bacterial resistance, further research is needed to achieve its safety and efficacy.<sup>81</sup>

## CRISPR-Cas System's Role in the Development of Antibiotic Resistance

### Relationship Between CRISPR/Cas and the Spread of Antimicrobial Resistance

The emergence and spread of antimicrobial resistance represents a threat to human health.<sup>82</sup> Antimicrobial resistance is mainly transmitted by HGT, leading to the spread of bacterial drug resistance.<sup>83</sup> CRISPR-Cas systems serve as genome defense systems that can defend against invading exogenous genetic material.<sup>84</sup> The structure and function of the CRISPR-Cas system are related to bacterial resistance. The CRISPR-Cas system can prevent the spread of plasmids and phages harboring antibiotic resistance genes, thus limiting the HGT of antibiotic resistance genes mediated by these mobile genetic elements<sup>16,85</sup> (see Figures 2 and 3B).

The ubiquitous CRISPR-Cas system in prokaryotes can be used to prevent and control the spread of antibiotic resistance.<sup>86</sup> Aydin et al<sup>87</sup> found that the type I CRISPR system in *Escherichia coli* may interfere with the bacteria acquiring the drug-resistant plasmids and maintain the sensitivity of the strain. A study by Price et al<sup>59</sup> demonstrated that CRISPR-Cas from mammalian intestinal flora can block the spread of antibiotic resistance plasmids in the mouse intestinal colonization model. Wang et al<sup>88</sup> show a negative correlation between the acquisition of antibiotic-resistant genes (ARGs) and the presence of CRISPR/Cas. It is worth noting that pathogens with CRISPR-Cas systems were less likely to carry antibiotic resistance genes than those lacking this defense system.<sup>89</sup> However, recent studies have shown that the CRISPR systems are sometimes missed or inactivated and may not be an effective barrier to plasmid and drug resistance spread.<sup>90</sup> Therefore, the analysis of the relationship between the CRISPR-Cas system and antibiotic resistance will help to better understand the mechanism of bacterial resistance and provide new directions for the prevention and treatment of bacterial resistance.

### CRISPR-Cas System's Role in the Spread of Antibiotic Resistance in Gram-Negative Bacteria

Gram-negative bacteria have a highly restrictive permeability barrier, and their low permeability of the outer membrane is a major cause of resistance to many antibiotics.<sup>91</sup> Infections caused by multiple drug-resistant (MDR) and widely drug-resistant (XDR) Gram-negative bacteria (GNB) have become a major challenge to public health.<sup>92</sup> The emergence and prevalence of drug-resistance genes such as *bla* KPC, *bla*NDM, *bla*VIM, and MCR-1 suggested that new therapeutic options for the treatment of MDR Gram-negative bacterial infections are urgently needed.<sup>93,94</sup> Previous studies have shown that the CRISPR-Cas system has been used to limit the spread of drug-resistant Gram-negative bacteria.

Carbapenems are the preferred treatment for severe infections caused by MDR and XDR Gram-negative bacteria (GNB).<sup>95</sup> Of particular concern is an increasing proportion of drug-resistant strains that are generated in Gram-positive bacteria such as extended-spectrum beta-lactamase (ESBL) and carbapenemase-producing *Enterobacteriaceae* (CPE). Hence, new treatment and prevention strategies are urgently required.<sup>96</sup> CRISPR-Cas9 technology can effectively eliminate carbapenem-resistant plasmids in pathogens and restore pathogen susceptibility to carbapenems.<sup>97</sup> The constructed CRISPR-Cas9 system was introduced into ESBL-producing *Escherichia coli* and the results indicated that this system can restore sensitivity to antimicrobial agents in the form of clearing resistance plasmids in ESBLs.<sup>64</sup> CRISPR-Cas9-mediated plasmid-curing system (pCasCure) can effectively remove carbapenemase genes (*bla* KPC, *bla*NDM, and *bla*OXA-48) and plasmids (*bla* KPC-harboring IncFIIK-pKpQIL and the *bla*NDM-harboring IncX3 plasmid, et al) in CRE isolates, thereby resensitizing CRE to carbapenems.<sup>97,98</sup> A study carried out by Kim et al,<sup>64</sup> where indicated that the CRISPR-Cas9 system targeting ESBLs-resistant plasmids was introduced into ESBLs-producing *Escherichia coli* constructed by transformation experiments (ESBLs-producing *Klebsiella pneumoniae* isolated from patients as plasmid

donors and *Escherichia coli* as plasmid recipients) and the results showed that the system could restore susceptibility to antimicrobials through eliminating ESBLs resistance plasmid. Wan et al<sup>99</sup> showed that horizontally transferable colistin resistance genes on MCR-1 plasmids can be knocked out using the CRISPR-Cas9 system.

CRISPR -Cas9 can simultaneously target multiple genes in the same single cell. The study by Vad-Nielsen et al<sup>100</sup> simultaneously targeted ten genomic loci and inhibited multiple endogenous genes by constructing a golden gate assembly of the CRISPR gRNA expression array. Further research achieved the simultaneous knockout of multiple  $\beta$ -lactamases with similar sequences in *Escherichia coli* by CRISPR-Cas, thus solving the problem of high diversity of  $\beta$ -lactamases to a certain extent.<sup>101</sup> It has been well reported in the literature that the CRISPR-Cas9 system simultaneously targets two super drug-resistant genes (MCR-1 and blaNDM-1), so that these two drug-resistant genes can be eliminated at the same time, ensuring the efficacy of carbapenem and colistin antibiotics.<sup>102</sup>

### CRISPR-Cas System's Role in the Spread of Antibiotic Resistance in Gram-Positive Bacteria

Gram-positive bacteria have thick cell walls, which can cause clinical infections of various diseases.<sup>103</sup> The emergence of drug-resistant Gram-positive bacteria has raised concerns, and methicillin-sensitive *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci* (VRE), and highly resistant strains to *Streptococcus penicillin pneumoniae* infections are a growing concern to human health.<sup>104</sup> As a gene defense system, CRISPR-Cas has been widely used to combat drug resistance in Gram-positive bacteria.

*Enterococcus faecalis* is a Gram-positive opportunistic pathogen that is the leading cause of nosocomial infections. Enterococcal infections are recognized as a serious public health threat, and the rise in antibiotic resistance makes these infections particularly difficult to treat.<sup>105,106</sup> Genetic analysis showed that CRISPR-Cas is a potent barrier to the horizontal acquisition of antibiotic resistance in *E. faecalis*.<sup>59</sup> The literature suggested an inverse relationship between the occurrence of the type II CRISPR-Cas system and antibiotic resistance in *E. faecalis*.<sup>107</sup> *E. faecalis* isolates generally possess only the orphan CRISPR2.<sup>108</sup> Most multidrug-resistant *E. faecalis* strains generally lack the functional CRISPR1-Cas or CRISPR3-Cas systems. The presence of the CRISPR-Cas system is associated with antibiotic sensitivity and lack of virulence features.<sup>109</sup> In *Enterococcus faecalis*, several studies<sup>110,111</sup> have revealed that the absence of antibiotic resistance was associated with the presence of CRISPR3. Differences in CRISPR1 loci have been reported among *E. faecalis* species, which may lead to the variability of CRISPR activity against antibiotic resistance genes in different species.<sup>112</sup> A study carried out by Rodrigues et al<sup>51</sup> where indicated that conjugative delivery of CRISPR-Cas9 can reduce the occurrence of antibiotic resistance in the *Enterococcal* population in a sequence-specific manner. Price et al<sup>12</sup> showed that orphan CRISPR2 loci requires the presence of CRISPR1-Cas derived from *Enterococcus faecalis* for genomic defense against MGE. As demonstrated by Gholizadeh et al<sup>113</sup> in studies with *Enterococcus faecalis* where they present a study showing that CRISPR-Cas can prevent the acquisition of some corresponding pathogenic factors in some isolates. In a study by Wu and coworkers found that the CRISPR-Cas9 systems targets the tetracycline resistance gene (tetM) and erythromycin resistance gene (ermB), respectively, successfully reducing antibiotic resistance to *E. faecalis* in vitro and in vivo.<sup>102</sup> These findings suggest that CRISPR-Cas systems can limit the spread of drug resistance in Gram-positive bacteria.

### CRISPR-Cas Systems' Role in Bacterial Biofilms

Bacterial pathogens often form biofilms, which helps them to resist different threats within the host.<sup>114</sup> Bacterial biofilms can increase the possibility of bacterial drug resistance and horizontal gene transfer.<sup>115</sup> The changes in the permeability of bacterial membranes can affect bacterial resistance.<sup>116</sup> The integrity of the bacterial envelope plays an important role in mediating antibiotic resistance and evasion of some inflammation caused by membrane-targeted antibiotics.<sup>117</sup> Studies found that the CRISPR-Cas system enhanced the envelope integrity by regulating bacterial lipoproteins, and ultimately cells provided the ability to resist membrane damage caused by antibiotics.<sup>81</sup> The CRISPR-Cas system is associated with the biofilm formation and colonization of the host.<sup>114</sup> In *Pseudomonas aeruginosa*, the type I-F CRISPR-Cas system has been revealed to inhibit biofilm formation by crRNA-guided targeting and destroying of prophage DNA.<sup>118</sup> Also, Zegans et al<sup>119</sup> demonstrated that the CRISPR system is required for bacteriophage DMS3-dependent inhibition of biofilm formation in *Pseudomonas aeruginosa*. In addition, studies by Sampson et al<sup>117</sup> revealed that the CRISPR-Cas endonuclease gene cas9 along with tracrRNA and ScaRNA represses the expression of bacterial lipoprotein (BLP),

thereby affecting envelope integrity-mediated antibiotic resistance and contributing to immune avoidance during infection in *Francisella novicida*.<sup>120,121</sup>

## CRISPR-Cas System's Role in Increasing Antibiotic Resistance

CRISPR-Cas systems may have a different effect on antibiotic resistance among different species.<sup>16,122</sup> Most studies showed that CRISPR-Cas system involvement in antimicrobial resistance.<sup>123</sup> However, several studies demonstrated that there was no significant relationship between the CRISPR-Cas system and antibiotic resistance in *Escherichia coli*.<sup>124</sup> Furthermore, Touchon et al<sup>125</sup> showed little effect of CRISPR on the epidemiology of plasmids in *E. coli* or on the spread of antibiotic-resistant genes. Type I CRISPR-Cas system not only plays a role in mediating anti-antibiotic resistance but also is associated with increased bacterial antibiotic resistance. For example, the I-F CRISPR-Cas system in *Pseudomonas aeruginosa* removed resistance genes or plasmids, thereby reducing the level of antibiotic resistance of the bacteria.<sup>126</sup> However, studies have suggested that the type I-E CRISPR system of *Vibrio cholerae* may facilitate the acquisition of bacteria carrying  $\beta$ -lactamase resistance genes.<sup>127</sup> Moreover, Shabbir et al<sup>128</sup> revealed that the CRISPR-Cas system promotes antimicrobial resistance in *Campylobacter jejuni* through transcriptome analysis. These results indicated that apart from the impact of limiting the spread of antibiotic resistance, the CRISPR-Cas system is associated with increased bacterial antibiotic resistance.

Previous studies indicated that *Klebsiella pneumoniae* strains with type I CRISPR system have a high number of tetracycline resistance genes, while they were more sensitive to aminoglycoside and  $\beta$ -lactam antibiotics and had fewer associated resistance genes.<sup>129</sup> A few resistant strains containing the CRISPR-Cas system were considered to be due to the mutation of the original spacer sequence, the partial or total deletion in the Cas gene cluster, and the presence of anti-CRISPR proteins.

## Anti-CRISPR (Acr) Proteins and Antibiotic Resistance

CRISPR-Cas system provides adaptive immunity in bacteria against diverse categories of phage infections and can defend against mobile genetic elements (MGEs).<sup>130</sup> Parallely, many phages and MGEs have evolved anti-CRISPR proteins (Acrs) to counteract the CRISPR-Cas system at different stages under the strong selective pressure exerted by CRISPR-Cas immunity.<sup>131</sup> Anti-CRISPR proteins (Acrs) disable CRISPR-Cas systems with diverse mechanisms. For example, AcrIF1, AcrIIA et al<sup>132–134</sup> can block DNA binding sites, and AcrIIC1, AcrIE1, AcrIF3, et al<sup>135–137</sup> can block DNA cleavage and even act enzymatically to disable CRISPR-Cas. Previous studies have shown that anti-type I CRISPR-Cas system ACPs such as AcrIE and AcrIF can inhibit the degradation of target DNA by preventing the recruitment of Cas3,<sup>137,138</sup> while anti-type II CRISPR-Cas system ACP can prevent from binding to target DNA by binding to Cas9.<sup>137,139–141</sup> Meeske et al<sup>142</sup> described that listeriophage ( $\phi$ LS46) encoding an anti-CRISPR protein (AcrVIA1) inhibits Cas13a by interacting with the guide-exposed face of Cas13a nuclease that prevents access to the target RNA and the activation of Cas13a RNase function and eventually inactivates the type VI-A CRISPR system. Prior studies have suggested that anti-CRISPR proteins, AcrF1 and AcrF2, inhibit the type I-F CRISPR/Cas system of *Pseudomonas aeruginosa* by preventing target DNA recognition. Shehreen et al<sup>143</sup> indicated that anti-CRISPRs might facilitate the uptake of ARGs in *Pseudomonas aeruginosa*. Anti-CRISPR (Acr) proteins carried by mobile genetic elements such as phages and conjugative plasmids demonstrate a role in the horizontal transfer of different MGE-encoded traits.<sup>139</sup> Anti-CRISPR promotes HGT by inhibiting CRISPR-Cas, and anti-CRISPR genes may be positively associated with antibiotic resistance. However, as pointed out by Stanley et al,<sup>144</sup> the phage encoding anti-CRISPRs remain sensitive to CRISPR-Cas, suggesting that anti-CRISPR action may be an imperfect process.

## Discussion

Antibiotic resistance is spreading rapidly around the world and poses a critical threat to public health. There is an urgent need for new strategies to control multidrug-resistant (MDR) bacterial infections and the spread of antimicrobial resistance.<sup>145</sup> The CRISPR-Cas system can specifically recognize and target the genetic elements carrying drug resistance genes or their transcripts and limit the spread of drug resistance genes, which shows great potential for preventing and controlling bacterial drug resistance.



As a gene-editing tool for adaptive immune defense against foreign nucleic acid invasion, the CRISPR-Cas9 system is more advanced than zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALEN), and other traditional gene-editing tools.<sup>146</sup> CRISPR-Cas shows many remarkable features of simple operation high efficiency, simple operation, good knockout effect, and low cytotoxicity.<sup>37</sup> Ding et al<sup>147</sup> used TALEN and CRISPR-Cas9 to modify the same gene, and the experimental results showed that the efficiencies were 0% to 34% and 51% to 79%, respectively, indicating the high efficiency of CRISPR. In addition, from an experimental point of view, CRISPR is easier to operate than TALEN, because each pair of TALEN needs to be resynthesized, but the design of Guide-RNA in the application of CRISPR technology is much simpler.<sup>148</sup> As a gene-editing tool, CRISPR/Cas9 technology, when combined with existing nucleic acid amplification technology, luminescence technology, rapid detection technology, etc., will show huge technical advantages such as high detection sensitivity, specificity, and reduced reaction time. For example, in 2017, *Science* published a molecular detection platform SHERLOCK based on Cas13a and recombinant polymerase amplification, which can detect Zika virus and dengue virus, identify mutations in circulating tumor DNA, etc., with extremely high sensitivity.<sup>149</sup> The specifically targeted site-specific cutting capability of CRISPR-Cas has made some achievements in nucleic acid testing and bacterial typing. These recent studies show that CRISPR technology has broad and rich application prospects in the field of medical laboratory.

In this review, we briefly introduce the structure, and mechanism of the CRISPR-Cas system, and the correlation of the CRISPR-Cas system with the spread of antibiotic resistance. Collectively, the effect of the CRISPR-Cas system on antibiotic resistance varies among different bacteria. The progress of introducing different types of CRISPR-Cas systems into plasmids, extracellular vesicles, phages, and nanoparticle vectors to reduce the level of bacterial drug resistance by targeting drug-resistant genes and plasmids was analyzed. Delivery of the CRISPR-Cas system or some of its components to bacteria still has some problems such as difficulty in the introduction and easy degradation by bacterial intracellular proteases or nucleases. Activation of ubiquitous endogenous CRISPR-Cas systems in bacteria using small molecule compounds or physicochemical factors may provide new directions for controlling the problem of bacterial multiple resistance due to drug resistance gene transfer.

The CRISPR-Cas system can limit the horizontal transfer of drug resistance genes in bacteria, and the application of bacterial genome programming provides important genetic tools for future research in the field of molecular biology. The CRISPR system has attracted more and more attention and become the research object of many scholars. Researchers have proposed and developed various efficient strategies to improve editing efficiency and reduce the off-target rate of CRISPR systems, and some remarkable results have been achieved. It is of great significance to utilize the CRISPR-Cas systems for the rapid detection of pathogenic microorganisms, the inhibition of viral infection, and the prevention and treatment of human infectious diseases. In the future, the CRISPR system will surprise us even more in terms of human health and medical development.

## Conclusion

CRISPR-Cas has the application prospect of preventing and controlling horizontal gene transfer and limiting the spread of antibiotic resistance. The CRISPR system can defend against the invasion of exogenous genes and is associated with bacterial drug resistance. Elucidating the structure and regulatory mechanisms of the CRISPR-Cas system and analyzing the relationship between CRISPR-Cas and bacterial drug resistance can help to better understand bacterial drug resistance and provide a new direction for controlling bacterial drug resistance. The CRISPR-Cas system has updated the understanding of bacterial function regulation, providing a new direction for the prevention and control of bacterial drug resistance and a means for effective immunotherapy in the medical industry.

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None of the authors has a conflict of interest to disclose.

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