Infection and Drug Resistance

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REVIEW

How CRISPR-Cas System Could Be Used to Combat Antimicrobial Resistance

This article was published in the following Dove Press journal:
Infection and Drug Resistance

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Abstract: Rapid emergence of antibiotic-resistant bacteria has made it harder for us to combat infectious diseases and to develop new antibiotics. The clustered regularly interspaced short palindromic repeats – CRISPR-associated (CRISPR-Cas) system, as a bacterial adaptive immune system, is recognized as one of the new strategies for controlling antibiotic-resistant strains. The programmable Cas nuclease of this system used against bacterial genomic sequences could be lethal or could help reduce resistance of bacteria to antibiotics. Therefore, this study aims to review using the CRISPR-Cas system to promote sensitizing bacteria to antibiotics. We envision that CRISPR-Cas approaches may open novel ways for the development of smart antibiotics, which could eliminate multidrug-resistant (MDR) pathogens and differentiate between beneficial and pathogenic microorganisms. These systems can be exploited to quantitatively and selectively eliminate individual bacterial strains based on a sequence-specific manner, creating opportunities in the treatment of MDR infections, the study of microbial consortia, and the control of industrial fermentation.

Keywords: antibiotic-resistant bacteria, CRISPR-Cas system, sequence-specific manner, resensitization, genome editing

Introduction

The extensive and often injudicious use of antibiotics in agriculture and public health over the last 7 decades has exerted a significant selection pressure for antibiotic-resistant bacteria to evolve. Various strategies against antibiotic-resistant bacteria have been introduced, including production of new antibiotics, bacteriophages that target and lyse these bacteria, and discovery and production of naturally and artificial-derived peptides or enzymes that specifically target genomes or functional vital proteins of these bacteria.[1,6] The widespread use and abuse of antimicrobial agents has driven bacterial populations to become resistant against antimicrobial agents through genetic additions and changes. The first global report by the World Health Organization (WHO) on antibiotic resistance in 2014 recognized that antibiotic resistance of bacteria is a serious threat to public health (https://www.who.int/mediacentre/news/releases/2014/amr-report/en/) with data collected from 114 countries. Interestingly, a small number of the pathogens are responsible for most antibiotic-resistant infections, which are highlighted by the Infectious Diseases Society of America, and they are known as ESKAPE pathogens. ESKAPE pathogens are a group of the most antibiotic-resistant bacteria, which cause difficulty in treating nosocomial infections. These pathogens include: *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* spp., which have or can acquire

resistance against multiple antibiotics.[7] The inherent difficulties in finding new antibiotics, along with the low economic motivation, have resulted in slow development of new antibiotics. Moreover, the pace of emerging antibiotic resistance far exceeds the pace of development of new antibiotics, which is insufficient to combat the rise in antibiotic resistance.[7] Therefore, new approaches have been suggested for decreasing and limiting antibiotic resistance in pathogens, including phage therapy,[2,8,9] use of peptide nucleic acid (PNA) as an ultra-narrow-spectrum antibiotic,[6,10,14] zinc finger nucleases (ZFNs),[15,16] and clustered regularly interspaced short palindromic repeat – CRISPR-associated (CRISPR-Cas) systems,[5,17,19] which are genomic engineering tools for gene knock-out and knock-in of sequence-specific DNA antibiotic targets. Bacteriophages are bacteria-specific viruses, which can specifically infect and lyse bacteria. Phage therapy harnesses phages for treatment against bacterial pathogens and their infectious diseases. Different clinical applications have been suggested for phages: 1) use of bactericidal and virulent phages against pathogens and antibiotic-resistant bacteria,[1,3,8,9,20] 2) the clinical use of metabolic inhibitor properties of phage structural proteins to inhibit bacterial cell-wall synthesis,[21] 3) the topical use of purified transglycosides and amidase encoded by phages as bacteriolytic cell-wall hydrolyses,[22,23] 4) use of the coated proteins of M13 phage for the phage-display system, which fused to specific antibodies against bacterial antigens,[24] 5) use of bacteriophages as a carrier and vehicles for the delivery of engineered genomic materials and vaccine antigens.[25,27] Owing to variable success and poor documentation of the use of phage therapy, the use of it has caused much of the controversy in the treatment of infectious diseases.[9]

However, most researchers focused on promising genes, which have potential targets for broad spectrum antisense growth inhibition in limited strains of one bacterial species or in different bacterial species.[6,10,12] Other antisense technologies, in addition to PNAs, using against resistant bacteria, are phosphorothioate oligodeoxynucleotides (S-oligos), locked nucleic acid (LNAs), and phosphorodiamidate morpholino-oligomers (PMOs).[28,30] Therefore, identification of gene targets for broad-spectrum antisense inhibition could help for the development of new antibacterial agents that could relieve the exacerbating clinical consequences caused by ESKAPE pathogens.

ZFNs and Transcription activator-like effector nucleases (TALENs) are restriction nucleases that can be engineered and designed to cleave specific sequences of DNA, and are also gene editing tools. These proteins are fused to a nonspecific endonuclease of the type IIS FokI restriction enzyme, which confers the nuclease activity of ZFNs and TALENs.[31,32] TALENs and ZFNs are similar to each other in that they can be used to knock-in or knock-out genes and generate double-strand breaks at a desired target site in the genome in the same way. However, the larger size of TALENs compared with ZFNs is a clear disadvantage, which makes it harder to deliver and express TALENs into cells.[33]

In all gene editing tools, the generated double-strand break can be repaired using either homology-directed repair (HDR) or non-homologous end joining (NHEJ) in the cells.[32] NHEJ is an error-prone mechanism that can knockout the gene by a combination of nonsense-mediated decay of the mRNA transcript and pre-mature truncation of the protein mechanisms, a process that is not always particularly efficient. In addition, HDR is another mechanism to repair double-strand break in DNA, by inserting a specific mutation with the introduction of a homologous piece of DNA. These mechanisms lead to mutations that terminate the translation of the gene product and change the open reading frame (ORF)[34,35] (Figure 1).

One of the most studied approaches that have been developed against antibiotic-resistant bacteria is the CRISPR-Cas systems. Several recent studies proposed CRISPR-Cas systems for controlling antibiotic-resistant strains.[5,17,36,40] Therefore, in this review article, we aimed to review the role of the CRISPR-Cas system to promote sensitization of bacteria against antibiotics.

Introducing CRISPR-Cas

CRISPR-Cas systems have been identified as a bacterial adaptive immune system[41] and as analogs of the mammalian immune system.[42] In the recent studies, the CRISPR-Cas system has been used for specific genome editing and different applications including treating genetic diseases,[43] genome engineering of various bacteria,[44] plants,[45] mice,[46] flies,[47] worms,[48] and more, and reversal of antibiotic resistance by targeting resistance genes,[5,17,40] as well as integration machinery of the systems, has been used to function as a molecular recording device.[49] This interesting system is found in approximately 50% of bacterial genomes and 87% of archaeal genomes.[50] The genetic loci of CRISPR-Cas systems contain the CRISPR array, which is comprised of short repeated sequences (repeats) and similarly sized flanking sequences (spacers). The spacers of CRISPR arrays are known as protospacers, which are acquired from DNA sequences from invading phage or plasmid. The Cas proteins are key functional elements of CRISPR systems, which are

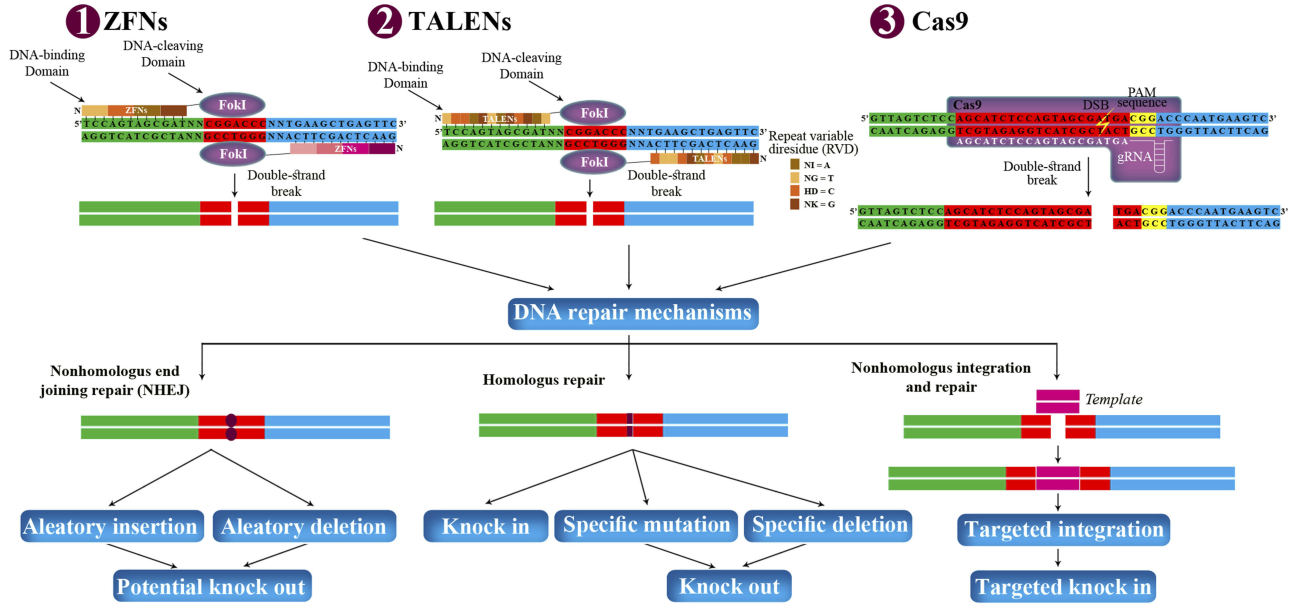

Figure 1 Potential genome manipulation using ZFNs, TALENs, and Cas9. ZFNs, TALENs, and Cas9 can be designed to target any gene in the genome of prokaryotic and eukaryotic cells. They are delivered to the cells via transduction or electroporation. Each ZFNs and TALENs contain non-specific DNA-cleaving domain (FokI), and DNA-binding domain. Each DNA-binding domain of ZFNs and TALENs recognizes 3–4 and 1 DNA sequence, respectively. Each repeat of TALENs is 33–35 amino acids in length, with two RVD (NI=A, NG=T, HD=C, and NK=G). The spacer regions between the monomers of TALENs and ZFNs are 6–40 bp and 5–7 bp in length, respectively. Dimerization of FokI is necessary for DNA cleavages within the spacer regions between the two bindings. In Cas9, gRNA recognizes and bind to targeted sequence, and Cas9 cleaves double-strand DNA in 3 bp upstream of the PAM sequence (NGG). The double-strand break can stimulate the natural DNA-repair mechanisms of the cell including nonhomologous end joining repair and homologous repair, followed by can be harnessed to create gene knock-out or knock-in. In addition, nonhomologous integration and repair is trended in biotechnology.

encoded upstream of the CRISPR array and determine the system activity.[50,51] The mechanism of CRISPR systems is similar to RNA interference (RNAi) in eukaryotic cells, which use small RNAs (sRNA) to identify and neutralize specific sequences of invading DNA including phages, transposons, and plasmids.[52,53] The mechanism of action of CRISPR-Cas systems is summarized in three stages including adaptation, expression, and interference.[54] During the adaptation stage, an approximately 30bp segment of homologous invading foreign DNA integrates into the leader side of the CRISPR locus, and protospacer adjacent motifs (PAMs) have been selected from spacers sequences of the host genome. During the expression stage, RNA is transcribed from the spacers of the CRISPR locus (pre-crRNA) and processed into crRNA. Ultimately, in the interference stage, crRNA along with Cas proteins specifically detect the invading DNA, cleave it, and generate a double-strand break.[54] There are six types of CRISPR-Cas system, which have been defined based on their sequence similarity, phylogenetic analysis, neighborhood analysis and comparison, distinct features of the components, and experimental data, including distinct features of the physiology, biochemistry, and molecular mechanism.[55] Based on the current classification in the Makarova et al[55] study, there are two classes of CRISPR-Cas system, which include six types (I–VI) and 33 subtypes. In this classification, Class 1 includes types I, III, and IV, along with 16 subtypes that contain multiple Cas proteins as effector modules, which form crRNA-binding complexes and mediate together in pre-crRNA processing and interference. Class 2 includes types II, V, and VI, along with 17 subtypes that contain a single, large, multidomain crRNA-binding protein (Cas9 in type II, Cas12 in type V, and Cas13 in type III), which is involved in all activities required for interference (in all variants), and in pre-crRNA processing (in some variants). Type I and II systems need two critical factors to effectively target DNA: 1) a PAM specific to each CRISPR-Cas system flanking the protospacer, and ii) complementarity between the target protospacer sequence and the CRISPR RNA spacer.[44,56,57] Effective targeting may occur even for multiple mismatches between the protospacer and the CRISPR RNA, although mismatches are more disruptive among the "seed" region flanking the PAM.[56,58] In type III systems, similar factors are required for DNA-targeting, where these systems assess base pairing between the region flanking the protospacer and the target sequence.[59] Therefore, the CRISPR system can be programmed to specifically detect any DNA target provided in the CRISPR array. CRISPR-Cas has been used to target specific genes

in virulence and genes that encode antibiotic resistance in bacterial populations.[17,37,39,60,63] Type IV systems are highly derived variants, which require the nucleases for interference and typically have no adaptation modules.[55] Other types of class 2 (ie, V and VI) contain large proteins as the effector modules, which clearly differentiate their domain structure.[64] The large effector protein of type VI and subtype V-A systems also contain RNase activity of the pre-crRNA processing, while this processing activity in type II and several subtypes of type V is typically relegated to RNase III, a non-Cas enzyme.[65,67] After this process, the crRNA-tracrRNA complex, as the mature guide RNA, allows for specific DNA interference through the remaining stable bound to the effectors.[68,71]

CRISPR-Cas Roles in the Antibiotic-Resistant Bacteria

In some studies, there was a significant reverse relation between the CRISPR-Cas system and antibiotic resistance in some species such as enterococci,[72,74] but, in other studies, there was no significant relation, such as in *E. coli*.[39] In enterococci, there are three CRISPR loci including CRISPR1-Cas, orphan CRISPR2, lack of *cas* genes, and CRISPR3-Cas.[74] Results have shown that orphan CRISPR2 is found in all *E. faecalis* strains, but the presence of CRISPR1-Cas and CRISPR3-Cas is varied among strains. In addition, Palmer and Gilmore[74] found that CRISPR1-Cas and CRISPR2 are functionally linked to each other. Analysis of CRISPR spacers demonstrated that pheromone-response plasmids play an important role in enterococcal genome plasticity, mobilizing chromosomally encoded virulence factors and antibiotic resistance, and are capable of promoting their own transfer, and causing displacement of CRISPR-Cas.[74,75] The distribution of these spacers of pheromone-responsive plasmids suggested that certain elements have a propensity to be incorporated into CRISPR loci as spacers or are frequently encountered by *E. faecalis*, but no CRISPR spacers have yet been identified against Tn*916*, as vectors of antibiotic resistance in enterococci.[76,77] The observation is that the *tetM* gene, which is commonly disseminated by Tn*916* and other conjugative transposons,[76,77] is present in *E. faecalis* possessing CRISPR loci,[74] which may suggest that conjugate transposons evade this defense. In addition, there is no spacer for the Inc18 plasmid family, which has disseminated vancomycin-resistance genes from enterococci to MRSA.[78,79] These findings may reflect the relative inefficiency of transfer of elements, which lack mechanisms for effective pair formation or the relative rarity of interspecies transfer. In addition, these roles were demonstrated in CRISPR-harboring strains of *Streptococcus thermophiles*, which acquired new spacers derived from the virus and became resistant to infection by phages.[41]

Brouns et al[53] reported that *E. coli* K12 strains, which carried an artificial CRISPR-Cas system with spacers that target genes of the Lambda phage, displayed decreased sensitivity to Lambda phage. In addition, Maraffini and Sontheimer[80] demonstrated that *S. epidermidis* may have plasmid conjugation limited by the CRISPR-Cas system, which suggests a broader and more critical role for the CRISPR-Cas system in the prevention of horizontal gene transfer (HGT). There are four CRISPR loci in *E. coli*, including CRISPR1, CRISPR2, CRISPR3, and CRISPR4, which each have a different type of *cas* gene.[81,82] Interestingly, all *Escherichia* genomes carrying CRISPR1 lack CRISPR4; consequently, no genome of *Escherichia* strains has more than three CRISPR.[82] Touchon et al[39] demonstrated that the presence of CRISPR loci of *E. coli* and the total number of repeats are not associated with the presence of integrons, plasmids, or antibiotic resistance. In addition, they assessed sequence similarities between available plasmid sequences and the spacers, and they found no evidence of spacers that matched elements involved in antibiotic resistance gene mobilization such as Tn*3, intI*, IS*Ecp1* or antibiotic resistance gene, ESBL production or the type of ESBL or replicon, and plasmid sequences.[39] They found only relatively small numbers of spacers matching plasmid genes among the *E. coli* strains examined and the spacers were specific for each group of CRISPR, including one single strain with 15 spacers; 77% of the strains had one, 13.2% had two, and 7.5% had three or four spacers. In addition, Touchon et al[39] found little effect of CRISPR on the epidemiology of plasmids in *E. coli* or on the spread of antibiotic resistant genes. These results are contrary to findings in enterococci, in which Palmer and Gilmore[74] found CRISPRs are inversely associated with antibiotic resistance. Based on these data, CRISPR-Cas systems may have a different effect on the antibiotic resistance among different species, which may be due to different evolutionary histories of the CRISPR-Cas system, CRISPR locus generation by partial or total deletion in the *cas* genes cluster, and the presence of anti-CRISPR proteins, just like Toxin-Antitoxin, or restriction and modification systems that are used for competition between plasmids.[82,85] When the *cas* system is complete, the number of repeats is high; when the erosion of the *cas* system is recent, the number of repeats is intermediate; and when only relics of the system are detectable, the number

of repeats is reduced to a few copies.[82] Interestingly, three very closely related strains of *E.coli* genomes, including BL21, BL21-DE3, and B-REL606 have a large number of repeats, which are devoid of *cas* genes, while a complete absence of CRISPR2 is detected in *E. coli* strains SMS35 due to a recent insertion of a sucrose operon.[82] Generally, the number of repeats is a good indicator of the possible functionality and integrity of the system. In addition, if the CRISPR locus contains spacers matching the chromosome, the host might pose a serious danger due to acquisition of a mobile element. If the CRISPR-Cas system triggers DNA plasmid degradation, which had spacers like chromosomal DNA, the outcome could be chromosome degradation. Also, if the CRISPR-Cas system only causes gene expression interference, this might still permit host manipulation by the foreign element. Therefore, bacterial genomes might have evolved a mechanism to inhibit or escape the incoming CRISPR-Cas system. This mechanism could be the use of native CRISPR acting as anti-CRISPR, which are demonstrated in *E. coli* and *P. aeruginosa*.[82,85]

CRISPR-Cas System Neutralizing Antibiotic-Resistant Genes

In general, RNA-based spacers that are flanked by partial repeats direct Cas proteins to specifically target DNA and cleave it that encode matching protospacers. Therefore, the CRISPR-Cas system can be programmed to specifically target and cleave any DNA for in vivo based on the information provided in the CRISPR array, which has been exploited to target the bacterial population which carry specific genes that encode antibiotic resistance.[8,60,63]

Recent work has shown that intentional or accidental targeting of the sequence of bacterial genome by the CRISPR-Cas system is cytotoxic, which can lead to cell death because of the introduction of irreversible chromosomal lesions.[61,86] Because of the widely conserved CRISPR-Cas system in bacteria and archaea, the isolation, optimization, and development of delivery carriers and vectors of the CRISPR-Cas system will be needed for the creation of RNA-guided nucleases capable of targeting additional strains, including multidrug-resistant (MDR) pathogens and key members of endogenous microbiota.[61] In addition, owing to the delivery system that acts in higher organisms, RNA-guided nucleases could enable us to modulate the prevalence of specific genes such as antibiotic resistance genes and virulence determinants in wild-type populations.[87] The Cas9 nuclease targeting specific DNA sequences of bacterial pathogens and antibiotic resistance gene are delivered to microbial populations using polymer-derivatized CRISPR nanocomplexes,[36] bacteria carrying plasmids transmissible by conjugation,[61,88] and/or bacteriophages[60,61] (Figure 2). Gomma et al[62] used the typed I–E CRISPR-Cas system of *E. coli*, encoding six *cas* genes in two operons including *casABCDE* and *cas3*,[53] to specific and essential sequences in the genomes of different sequences. They concluded that potent elimination can be achieved by targeting of multiple and divers locations including *ftsA, nusB, msbA*, and *asd* throughout the genome, and extents of elimination exhibited by simultaneous targeting of multiple locations similar to those with targeting of only one of the locations.[62] In this study, they used transformation in spite of using a potent procedure for delivering a type I CRISPR-*cas* system into bacteria, as well as targeting chromosomal genes, which are unique to part of the mixed population, in spite of targeting resistance genes on extrachromosomal elements. Bikard et al[60] exploited the Cas9 phagemid, which is a plasmid that is designed to be packaged in phage capsids,[89] and to eradicate MRSA strains from a mixed population of bacteria. In addition, they designed a CRISPR-Cas9 plasmid that targets tetracycline-resistant plasmids including pUSA01 and pUSA02. They are constructed *cas9* and crRNA of methicillin resistance gene *mecA*[90] in the phagemid (pDB121::*mecA*), to treat the clinical isolate of *S. aureus* USA300$^{\Phi}$. In all cases of Cas9 plasmid that target tetracycline-resistant plasmids, cell death was not observed, but tetracycline sensitivity was found in more than 99.99% of the cells, and a decrease in proportion of *S. aureus* USA300$^{\Phi}$ was observed from 50% before treatment to 0.4% after treatment of pDB121:*mecA* phagemid. In addition, they constructed enterotoxin *sek* gene[91] in the phagemid and they observed the phagemid able to kill all *S. aureus* with comparable efficiencies. In another study, Citorik et al[61] used two approaches including M13-based phagemid and conjugative plasmid to deliver the Cas9 nuclease into bacteria for targeting $bla_{\text{NDM-1}}$ and $bla_{\text{SHV-18}}$, which encode pan-resistant to beta-lactams and extended-spectrum resistant to antibiotics, respectively.[92,93] Treatment of the resistant strains with the phages resulted in 2–3-log reductions in viable cells. In addition, they demonstrated that the CRISPR-Cas system could discriminate between resistant and susceptible strains, which found that programmed *gyrA* phagemid was specifically cytotoxic only for chromosomal *gyrA* mutations that are responsible to quinolone-resistant *E. coli*[94] and not cytotoxic for isogenic strains with the wild type *gyrA* gene.[61]

In another study, Kang et al[36] introduced a nonviral delivery method based on a Cas9-nanocomplex, which

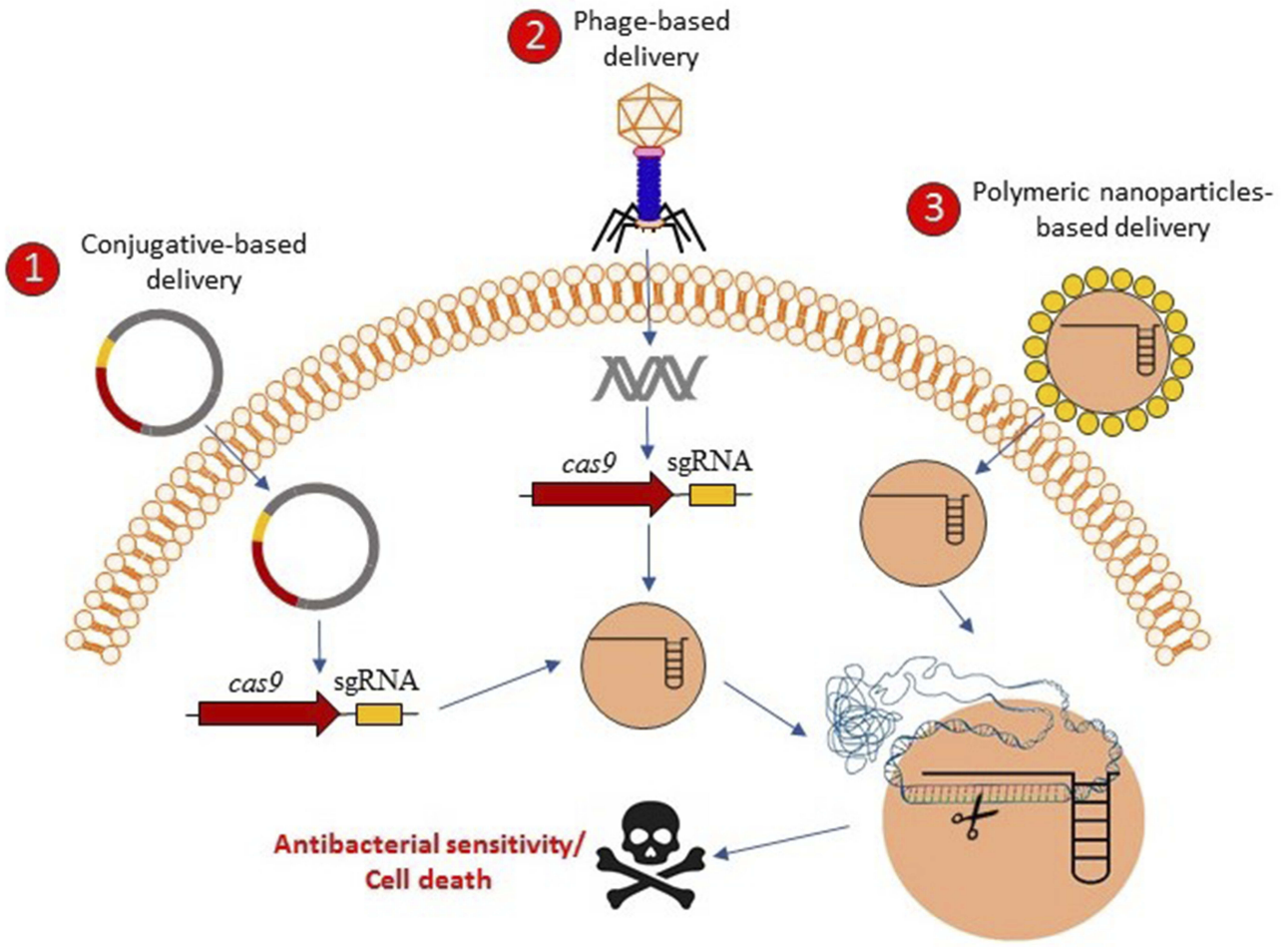

Figure 2 Graphical concepts model of CRISPR-Cas delivery for antibacterial affecting. CRISPR-Cas-based antibacterials could be delivered into the bacterial cells through the three proposed delivery mechanisms including 1) conjugative-based delivery, 2) phage-based delivery, and 3) polymeric-nanoparticles-based delivery. After the delivery of CRISPR-Cas systems in the bacterial cells, the bacterial cells might be resensitized against antibacterial agents or be killed based on the antibacterial resistant genes targets or essential genes targets, respectively.

used Cas9, sgRNA targeting *mecA*, and a cationic polymer, known as branched polyethyleneimine (bPEI). The bPEI is one of the most commonly used carriers for gene delivery[95,96] and was used as the carrier for packaging sgRNA, which can enhance Cas9 delivery into MRSA strains.[36] Kang et al[36] demonstrated that Cas9 conjugated with bPEI could uptake into the bacteria greater than native Cas9 simply mixed with bPEI and native Cas9 mixed with lipofectamine (a carrier for gene delivery in mammalian cells[97,98]), which did not show any sign of uptake. They suggested that this phenomenon could be due to the resultant enhancement in polarity of the protein or highly cationic characteristic of the bPEI polymer.[36] In addition, the cultured MRSA strains treated with Cas9-bPEI could not be able to grow in agar media, including 6 μg/mL oxacillin, while the strains that were not treated could grow in the media. Also, treatment with the Cas9-bPEI could significantly decrease the growth (a 32% decrease) compared to treating with the Cas9-bPEI complex without sgRNA as the control, while native Cas9/sgRNA complexed with a lipofectamine carrier showed no decrease in growth.[36] This finding could be a forward step to design CRISPR-based antimicrobials drugs, because vector-free delivery of CRISPR can avoid off-target effects and immunogenicity problems, as well as could simply induce phenotypic changes and edit the bacterial genome.

One of the limiting factors in CRISPR-mediated killing by plasmids is the low frequency of conjugation, which can be developed phagemid- or bacteriophage-mediated delivery that are much more efficient. Nonetheless, delivery of CRISPR nucleases by the conjugative plasmid delivery remains an interested option due to conjugative plasmids which do not require a cellular receptor,[99] are

easy to engineer with large coding capacities,[100] are resistant to restriction-modification systems,[101] and have broad host ranges.[102] Conjugative plasmids that encode and promote biofilm formation could increase rates of conjugative plasmid transfer due to the enhanced cell-to-cell contact,[103] which may be suited for delivery of molecular tools such as CRISPR nucleases for modulating composition of microbial communities[104,106] that many of them exist as biofilms. In a study by Hamilton et al,[107] they developed a cis-conjugative system that the plasmid encodes both CRISPR nuclease and conjugative machinery. They designed 65 total sgRNA, which target 38, 23, and 4 essential genes, non-essential genes and genes with unresolved phenotypes. They found that plasmids containing conjugative machinery and CRISPR nuclease under conditions that enhance cell-to-cell contact have a higher frequency of conjugative transfer from *E. coli* to *Salmonella enterica*. They concluded that single or multiplexed sgRNAs targeting non-essential genes such as *katG* (catalase reductase), *yghJ* (putative lipoprotein), *aegA* (putative oxidoreductase), and *gltJ* (glutamate/aspartate transporter) result in high killing efficacy of *S. enterica* compared to essential genes. In addition, they demonstrated that when the bacteria receive the cis-conjugative plasmids, recipients become potential donors for subsequent rounds of conjugation and could be potentially leading to exponentially enhancing numbers of conjugative donor bacteria in the population. They suggested that this delivery system combined by CRISPR nucleases could be an effective tool for modification of microbiome.

Citorik et al[61] demonstrated that treatment of enterohemorrhagic *E. coli* (EHEC) with phagemid *eae* targeting intimin, which is a chromosomally encoded virulence factor of *E. coli* O157:H7 and is necessary for intestinal pathology and colonization,[108] resulted in a 20-fold reduction in viable cell counts. These data demonstrated that the CRISPR-Cas system activity can selectivity remove bacteria with specific genome, which could reduce the prevalence of unwanted genes, such as virulence loci, antibiotic resistance, or metabolic pathways from the bacterial population without affecting bystanders. In addition, the CRISPR-Cas system can be used to modulate the composition of complex bacterial communities, which current therapies that use a drug to modify the human microbiota, prebiotic or probiotic have potential for reducing suffering of various diseases, but such therapies remain poorly characterized in terms of the specific mechanisms by which they act.[109]

The efficiency of the Cas9 phagemid against pathogens were tested in an in-vivo mouse model.[60] Topical treatment of the back of CD1 mice, which were infected by RNK^{Φ} cells, with the CRISPR-Cas9 antimicrobial pDB121::*aph* phagemid (against kanamycin resistance gene *aph*[110]), demonstrated a decrease in the proportion of RNK^{Φ} cells that was significantly different from 2% mupirocin or streptomycin (200 mg/mouse) treatments ($P<0.0001$), of which streptomycin treatment eliminated the infection, but mupirocin did not.[60] In another study, Citorik et al[61] demonstrated that treatment of EHEC with *cas9-eae* phagemid in *Galleria mellonella* larva was significantly improved survival over no treatment control and was significantly more efficient than chloramphenicol treatment, to which the strain was resistant, as well as was inferior to carbenicillin, to which the strain was susceptible. *G. mellonella* is an infection model to evaluate the efficiency of phage therapy and antimicrobials against various Gram-positive and -negative and fungal pathogens.[111] Other studies, such as Kiga et al,[112] designed Cas13a-based phage to target carbapenem-resistant *E. coli* and methicillin-resistant *S. aureus*. These data support the CRISPR-Cas system and use of phagemid as viable alternatives for cases where bacterial strains are highly resistant to existing antimicrobial agents.

However, the use of phagemid has two key weaknesses. First, the phagemid does not produce more phages after infections, which means that the amount of phagemid needed to treatments are much larger than the size of the target population. Second, the narrow-host range and large scale population of phagemids could preclude their extensive use.

The advantage of programmed Cas9-mediated killing is the possibility of a nuclease with two or more crRNA guides to trigger different plasmid and/or chromosomal sequences, which could decrease resistant clones that desert phagemid treatment through the generation of target mutations, as well as extend the range of targeted cells. In addition, delivery of the sequence-specific Cas9 nuclease and easy reprogramming to target different sequences reduces the plasmid content in a bacterial population without killing the cells, which can be immunized non-pathogenic strains against the transfer of antibiotic-resistant and/or virulence plasmids.

As future directions, depending on the nature of the dysbiosis and microbiome, most of the researchers focused on the phage-, conjugative-, and polymeric nanoparticles-based CRISPR delivery systems and the best killing efficiency target genes. Most of the studies suggest that a combination of the phage-, conjugative-, and polymeric

nanoparticles-based delivery systems may be appropriate for planktonic and biofilm conditions. In addition, a central problem in microbiome manipulation, microbiology, and infectious disease control is complete elimination of the target organism(s) and is the lack of special tools to control pathogenic species or to alter the composition of microbial communities.[113] CRISPR-based nucleases as sequence-specific antimicrobial agents could reduce the relative abundance of the target and infectious bacteria, yet the development of a broadly applicable and robust delivery system remains a key milestone. Therefore, the challenges ahead against CRISPR-Cas-based antibacterials could be CRISPR-Cas delivery vectors or vehicles and architecture of them, complex bacterial communities, different mechanisms of resistance to one antibacterial agents in the organism(s), possibility of mutations in the target genes, legislation, and social responsibilities of CRISPR-Cas-based antibacterials.

Conclusion

Owing to the rational design, sequence-informed and additional of facile to a field that has been dominated by cost- and time-intensive screening for broad spectrum, the small-molecules and CRISPR-Cas-based antibiotics have the potential to reinvigorate novel horizons of development for new antimicrobials.

Modern antibiotic therapy has shifted toward the straightforward acquisition of antibiotic resistance genes, among other things, increasing plasticity or decreasing genome stability, and enabling the colonization of new habitats such as the antibiotic-laden hospital environment.

We envision that CRISPR-Cas approaches may open novel ways for the development of smart antibiotics, which can eliminate MDR pathogens and make differentiation between beneficial and pathogenic microorganisms. These systems can be exploited to quantitatively and selectively eliminate individual bacterial strains based on the sequence-specific manner, creating opportunities in the treatment of MDR infections, the study of microbial consortia, and the control of industrial fermentations.

Ethics and Consent Statement

All figures in this study are original and not reproduced from any other study.

Acknowledgment

We thank all staff of the DARC research center for all their support and collaboration. We also thank Imam Reza Hospital staff, Rohollah Safari, Laleh Jamali, and Ms Kalani, who helped us to get our goals for infection control.

Funding

This study was supported by Iran National Science Foundation (INSF) with the grant number 97015174 and Faculty of Medicine, Tabriz University of Medical Sciences.

Disclosure

The authors report no conflicts of interest in this work.

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