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REVIEW

Recent Advances in Understanding the Molecular Mechanisms of Multidrug Resistance and Novel Approaches of CRISPR/Cas9-Based Genome-Editing to Combat This Health Emergency

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Abstract: The rapid spread of multidrug resistance (MDR), due to abusive use of antibiotics has led to global health emergency, causing substantial morbidity and mortality. Bacteria attain MDR by different means such as antibiotic modification/degradation, target protection/modification/bypass, and enhanced efflux mechanisms. The classical approaches of counteracting MDR bacteria are expensive and time-consuming, thus, it is highly significant to understand the molecular mechanisms of this resistance to curb the problem from core level. The revolutionary approach of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated sequence 9 (CRISPR/Cas9), considered as a next-generation genome-editing tool presents an innovative opportunity to precisely target and edit bacterial genome to alter their MDR strategy. Different bacteria possessing antibiotic resistance genes such as *mec*A, *erm*B, *ram*R, *tet*A, *mqr*B and bla_{KPC} that have been targeted by CRISPR/Cas9 to re-sensitize these pathogens against antibiotics, such as methicillin, erythromycin, tigecycline, colistin and carbapenem, respectively. The CRISPR/Cas9 from *S. pyogenes* is the most widely studied genome-editing tool, consisting of a Cas9 DNA endonuclease associated with tracrRNA and crRNA, which can be systematically coupled as sgRNA. The targeting strategies of CRISPR/Cas9 to bacterial cells is mediated through phage, plasmids, vesicles and nanoparticles. However, the targeting approaches of this genome-editing tool to specific bacteria is a challenging task and still remains at a very preliminary stage due to numerous obstacles awaiting to be solved. This review elaborates some recent updates about the molecular mechanisms of antibiotic resistance and the innovative role of CRISPR/Cas9 system in modulating these resistance mechanisms. Furthermore, the delivery approaches of this genome-editing system in bacterial cells are discussed. In addition, some challenges and future prospects are also described.

Keywords: bacteria, multidrug resistance, CRISPR/Cas9, gene editing, nanoparticle, delivery approaches

Introduction

Since the discovery of antibiotics in 1928, commonly used as a weapon of choice against infections, the frequency of antibiotic-resistant infections are increasing rapidly. The widespread use and misuse of antibiotics, inadequate surveillance and regulations has led the bacteria to evolve into antibiotic-resistant strains.[1] Some factors like inappropriate and repeated use of similar antibiotic sets, demography, lifestyle and host biology play a significant role in sensitivity of resistance leading to enhancement in antimicrobial resistance (AMR).[2,3] Consequently, the therapeutic efficacy of antibiotics is significantly reduced by pathogenic AMR and it has led to the evolution of multidrug resistance (MDR) pathogens.[4,5] Therefore, the eradication of MDR complication is utmost important by developing some novel therapies.

MDR is a global health emergency, leading to a huge financial burden, causing substantial morbidity and mortality, threatening to undermine the practice of medicine.[6,7] As per the center for disease control and prevention (CDC) records, there is an urgent threat from drug-resistant bacteria, which include Carbapenem-resistant *Enterobacteriaceae* (CRE), Carbapenem-resistant *Acinetobacter baumannii*,[8] *Clostridioides difficile* (*C. difficile*) and Drug-resistant *Neisseria gonorrhoeae* (*N. gonorrhoeae*).

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Some other serious threat bacteria include extended-spectrum beta-lactamase, drug-resistant (*Campylobacter, Streptococcus pneumoniae, Shigella, Salmonella serotype typhi, M. tuberculosis*, and non-typhoidal Salmonella). In addition, other bacteria in this category include (ESBL)-producing *Enterobacteriaceae*, multidrug-resistant *Pseudomonas aeruginosa*, (*P. aeruginosa*), vancomycin-resistant *Enterococci* (VRE), and methicillin-resistant *Staphylococcus aureus* (MRSA). While the concerning threat strains also include Clindamycin-resistant group B *Streptococcus* and Erythromycin-resistant group A *Streptococcus*.[9] Even though, not all bacterial groups are resistant to antibiotics, six major MDR bacteria, commonly known as ESKAPE bugs are identified which mostly evade the antibiotics action. These include *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumonia, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter species* and these bacteria are mainly responsible for global nosocomial infections.[10,11]

Different approaches have been practiced time to time to combat the problems of AMR. These approaches include the use of vaccines, drug repurposing, antimicrobial peptides, phage therapy, anti-virulence compounds, etc. Each of these strategies has some unique features but also suffers from some limitations, thus pushing the researchers to explore some new alternatives with enhanced efficiency. The strategy of RNA interference, restriction endonucleases, and other genome-editing tools have also been practiced recently to combat the problems of MDR.[12]

The recent breakthrough towards combating the AMR has led to the use of Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated sequence (CRISPR/Cas), primarily a prokaryotic immune system, to overcome the problems of antibiotic resistance challenges.[13] CRISPR/Cas system are well-known genome-editing defense systems with different classes, types and subtypes, present in prokaryotes and archaea against mobile genetic elements, plasmids and phages. The discovery of the CRISPR/Cas system has been a major breakthrough in genome-editing technology. This tool allows the bacteria to be specifically modulated genetically for its virulence or resistance.[14] Among all CRISPR/Cas types, CRISPR/Cas9 possess some special features, due to its simple structure, high versatility and is thoroughly studied as a genome-editing tool, thus making it highly suitable for MDR applications.[15]

In this review, the recent updates of some molecular mechanisms of AMR are explored. In this context, the novel approaches of CRISPR/Cas9 as a genome-editing tool to diminish the MDR are discussed. In addition, the delivery approaches of this genome-editing tool to different bacteria are demonstrated. Furthermore, some challenges and future prospects of MDR is also elaborated.

The Biology of Antibiotic Resistance

The ability of bacteria to survive in antibiotics environment, expected to kill them is known as antibacterial resistance or MDR. Even though the MDR is increasing day by day, an increasing expertise about the biological insights and technological advances are also developing in parallel. Bacteria use either an "intrinsic" mechanism or an "acquired" resistance approach for their survival in different antibiotic conditions.[16] The resistance mechanisms involve the resistance genes expression against different targets, which can markedly change between varying growth conditions.[17]

Bacteria often get mutations in their DNA by phages, plasmids and other mobile genetic elements (MGEs).[18] The MDR is broadly classified into two types as genetic MDR and phenotypic MDR. The mutations in bacterial DNA or antibiotic resistance genes received from other bacteria give rise to genetic MDR. Clinically, this type of MDR leads to primary treatment failure thus requires alternative antibiotic use or different therapeutic approaches. In contrast to genetic MDR, phenotypic MDR results in changes in bacteria and subside within individual cells, without genetic alteration. This type of MDR against a specific antibiotic does not allow overall growth of the whole bacterial population (Figure 1).

The growth of the overall population of bacteria is not allowed in the case of phenotypic MDR in the presence of a specific antibiotic at or above minimal inhibitory concentration (MIC). This type of AMR can manifest as a minimal killing rate for the bulk bacterial population, sometimes termed as "tolerance".[19] Phenotypic antibiotic resistance, alternatively have a slower killing rate than most of the bacterial population, often termed as "persistence".

A thorough knowledge about the molecular mechanisms of MDR can help to discover some innovative approaches to treat infectious diseases.[20] Recently, considerable progress has been made to understand the antibiotic action and the bacterial inhibitory resistance mechanisms against the killing effects of these antibiotics. The antimicrobial resistance by bacteria is employed by three related approaches, which include tolerance, resistance and persistence.[1]

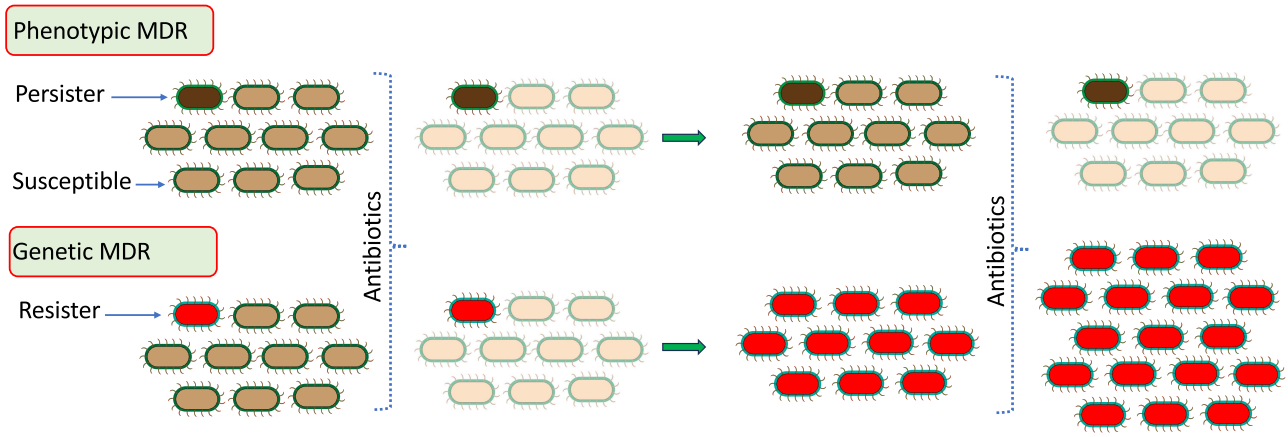

Figure 1 Diagrammatic representation of phenotypic and genetic MDR. There exists a genetic identity between phenotypic MDR bacterial cell and its siblings, but the metabolic state of this cell is conductive to survive the first exposure of a specific antibiotic (dark grey cell). However, this bacterium makes its new progeny so that the second exposure of a given antibiotic kills the same proportion of bacteria as before. In genetic MDR, a mutated bacterium (red cell) has the potential to resist antibiotic concentration to survive, and the division of these bacteria continues. Under the exposure of specific antibiotics, majority of the susceptible bacteria (light grey cells) die. The proliferation of resistant bacteria continues and the new progeny keep on having the induced mutation, even in absence of a specific antibiotic. A second exposure of the same antibiotic does not affect the bacterial survival, which continue to grow.

There has been a significant advancement in understanding the biochemical action of different antibiotics and the major mechanism by which bacteria resist the killing potential of antibiotics through different ways. The resistance mechanisms include diverse actions, such as expression of resistance genes, downregulation or modification of porins to decrease the antibiotic influx, antibiotic inactivation, overexpression of active efflux pumps, antibiotic target site modification, target bypass, and target protection (Figure 2).

Recent advances in different technologies have revealed precise details involved in diverse resistant mechanisms. These include the action of complex efflux systems pointing towards probable routes for inhibitor development.[21,22] The

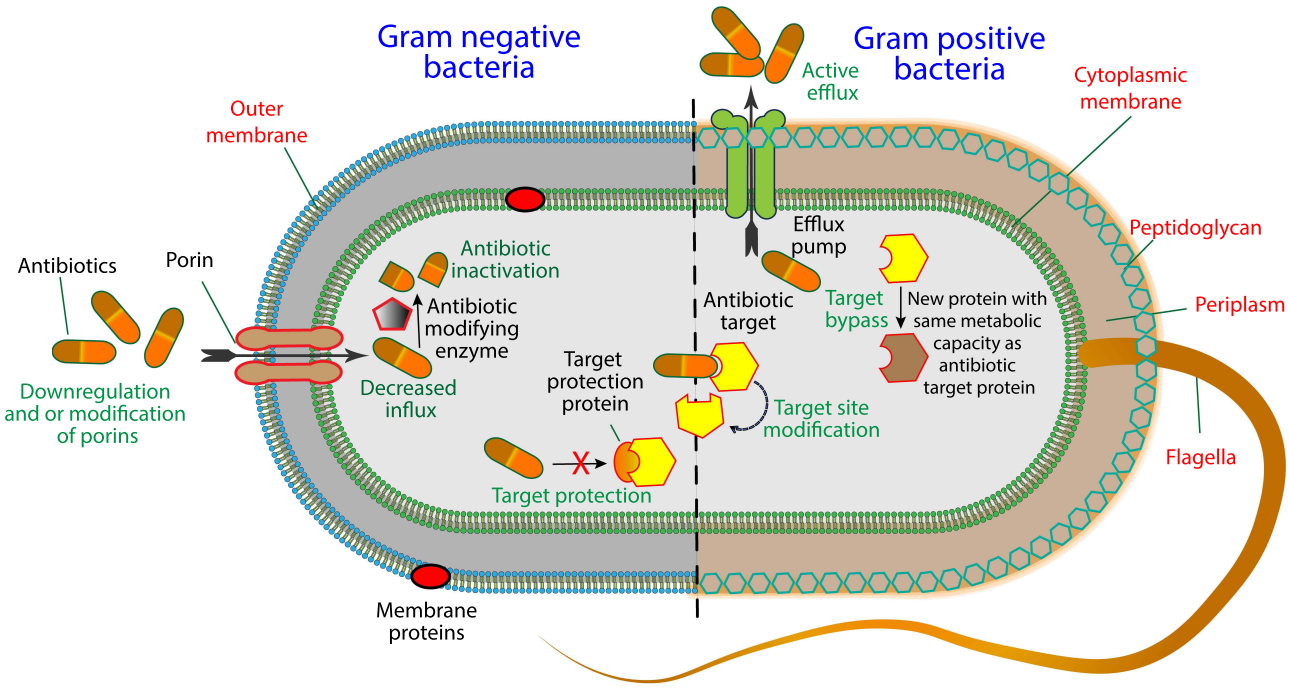

Figure 2 Overview of different molecular mechanisms of MDR in bacteria. The downregulation of transmembrane proteins or changes in their structure lead to decreased influx of antibiotics. The number or activity of transmembrane efflux pumps increased, exporting the antibiotics out of the cell to minimize their intracellular concentration. Some of the enzymes either modify or degrade antibiotics by hydrolysis or transfer of some chemical groups and thereby rendering it ineffective. The target site alterations of specific proteins by mutation of genes encoding the protein target or changes in binding site. The target bypass involves the binding of antibiotic to a new protein, without inhibiting its activity. A physical association of target protection proteins with antibiotic target proteins relieve it from antibiotic-mediated inhibition.

mechanism of antibiotic resistance with selected examples of different proteins and enzymes and their gene location is summarized in Table 1.

Different Approaches to Overcome MDR

Different approaches to curb the MDR include the use of combinatorial therapy, vaccines, drug repurposing,[44,45] antibodies, antimicrobial peptides,[46,47] phage therapy,[48,49] anti-virulence compounds,[50] and drug loaded nanoparticles (NPs).[51–53] Each of these strategies faces some limitations, forcing the exploration for new alternatives with enhanced efficiency.

Combinatorial therapy comprises the practice of using different drug combinations rather than single drug to attain the synergistic effect to kill the bacteria by targeting multiple sites. The rapid occurrence of MDR bacterial strains necessitates the use of combinatorial therapy rather than monotherapy, which is increasingly no longer adequate to threat most of the bacterial infections.[54] However, due to some incompatibility complications between different drugs, this procedure affects the pharmacokinetics and pharmacodynamics of the used drugs.[55,56]

Phage therapy has proven to be efficient to some extent against antibiotic resistant bacteria.[57] However, some challenges include their interaction with intracellular bacteria, promote neutralizing antibodies production and the development of phage resistance in bacteria.[58] Some bacterial resistance has also been treated with the use of monoclonal antibodies (mAbs). However, some barriers impeding their use include bacterial target selection (eg, lipopolysaccharides possess different serotypes), and degradation by bacterial proteolytic enzymes.[59]

The approach of RNA interference (RNAi) and steric-blocking oligonucleotides are some RNA based therapeutics used to treat AMR organisms. These approaches exploit the use of oligonucleotides to target bacterial mRNA enzymatically, allowing the removal of genes conferring the resistant phenotypes.[60] Antisense RNA-based procedures have also offered the means to keep the track of genes responsible for MDR and growth promotion.[61,62] However, RNA-based therapeutics suffer from poor intracellular uptake and some toxicity issues.[60]

The MDR complications have also been sorted out by the use of gene editing tools like Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) that can be used to precisely edit DNA of drug resistant bacteria. The employment of TALENs and ZFNs is used against specific DNA sequence for their cleavage.[63,64] The TALENs and ZFNs tools have paved novel approach of modern gene editing strategy. Due to some substantial limitations, such as complexity, off-target

Table 1 Different Mechanism of Attaining MDR by Bacteria, Selected Examples and Gene Locations

Mechanism of MDR	Selected Examples	Gene Location	Reference
Antibiotic modification/ degradation	β-lactamases Class A, B, C	*Streptomyces* species chromosome	[23]
	ANT Streptomycin-6 phosphotransferase, AME:AAC; APH	*S. griseus* (*smk*) chromosome	[24,25]
Antibiotic efflux	MFS transporter OtrB (oxytetracycline) Mfs1 (natamycin)	*S. rimosus* (*otr*B) *S. chattanoogensis* (*mfs1*) chromosome	[26–28]
	OtrC (oxytetracycline) ABC transporter DrrAB (Dox)	*S. peucetius* (*drr*AB) *S. rimosus* (*otr*C) chromosome	[29,30]
Antibiotic target modification	Sgm methylase, 16S rRNA methylation (Aminoglycosides) PCT	*S. pactum* (*pct*) *M. zionesis* (*sgm*) chromosome	[31,32]
	PikR1, PikR2, 23S rRNA methylation (MLS) Clr	*S. caelestis* (*clr*) *S. venezuelae* (*pik*R1, *pik*R2) chromosome	[33,34]
	Low affinity PBP Class A Class B	*Streptomyces* species chromosome	[35,36]
	Peptidoglycan remodeling (Glycopeptides) $VanH_{st}$, $VanX_{st}$ $VanH_{aov}$, DdlM, $VanX_{aov}$, DdlN	*A. orientalis* (*van*H_{aov}, *ddl*N, *van*X_{aov}); *S. toyocaensis* (*van*H_{st}, *ddl*M, *van*X_{st}) chromosome	[37,38]
Antibiotic target protection	Antibiotic removal DrrC (Dox) OtrA (oxytetracycline)	*S. peucetius* (*drr*C) *S. rimosus* (*otr*A) chromosome	[39,40]
Antibiotic target bypass	DNA gyrase subunit B (novobiocin)	*S. sphaeroides* (*gyrB*^{R}) chromosome	[41]
Antibiotic sequestration by special proteins	Sequestration TlmA, ZbmA (bleomycin), BlmA	*S. verticillus* (*blm*A); *S. hindustanus* (*tlm*A); *S. flavoviridis* (*zbm*A) chromosome	[42,43]

Abbreviations: AME, Aminoglycoside modifying enzyme; AAC, N-acetyl transferases; APH, O-phosphotransferases; ANT, O-adenyltransferases; ABC, ATP-binding cassette superfamily; MFS, major facilitator superfamily; Dox, doxorubicin; MLS, macrolides, lincosamides, and streptogramins; PBP, Penicillin-binding protein; PCT, pactamycin methylase; Sgm, sisomicin-gentamicin resistance methylase.

effects and delivery challenges, these genome-editing approaches have not gained a broad range success. These strategies finally led to the novel use of CRISPR/Cas-based genome-editing for the antibiotic resistance.

Currently, the CRISPR/Cas system is considered as the most innovative approach of genome editing and has been rapidly used for the management of MDR since its discovery. It is extensively used as a genome editing approach since it is fast, less expensive and the most efficient genome-editing tool. It is also used for the removal of bacterial pathogens,[65,66] elimination of major infectious viruses,[67,68] and improvement of genetic defects.[69,70] Different scientific studies support the control of spread of MDR via the use of CRISPR/Cas-based strategy.[68,71,72]

CRISPR/Cas System and Its Types

The CRISPR/Cas system is an innovative genome-editing tool, which acts as an adaptive immune system in archaea and bacteria protecting them from phages, plasmids and other mobile genetic elements. Almost 75% of archaea and 36% of bacteria possess the CRISPR/Cas system in their genome. Recently, it has been reported that there exists an inverse association between the existence of CRISPR/Cas system with antibiotic resistance, ESBL and carbapenemase production in antibiotic resistant bacteria, such as *K. pneumonia*.[73] In addition, the prevalence of CRISPR/Cas system and its possible association with antibiotic resistance has been reported in *E. faecalis* and *E. faecium*.[74]

The CRISPR/Cas genetic loci integrate the CRISPR array, compromised of repeated sequence and short flanking sequences (spacers). Protospacers constitute the spacers of CRISPR, derived from invading plasmids or phage DNA. Cas proteins, the key components of the CRISPR system, are encoded towards the upstream of the CRISPR array.[75,76] The CRISPR array may be clustered on a chromosome in numerous or single loci.[77,78] In 2012, there was a great achievement in this genome-editing system when Doudna and Charpentier et al, used CRISPR/Cas9 system having chimeric single guide RNA (sgRNA). The sgRNA is customized by joining CRISPR RNA (crRNA) and transactivating CRISPR RNA (tracrRNA) together.[79]

The CRISPR/Cas systems are grouped in two classes, six types (type I to type VI) and 33 subtypes. The classification of this genome-editing tool is based on differences in Cas protein content and is briefly summarized in Figure 3.

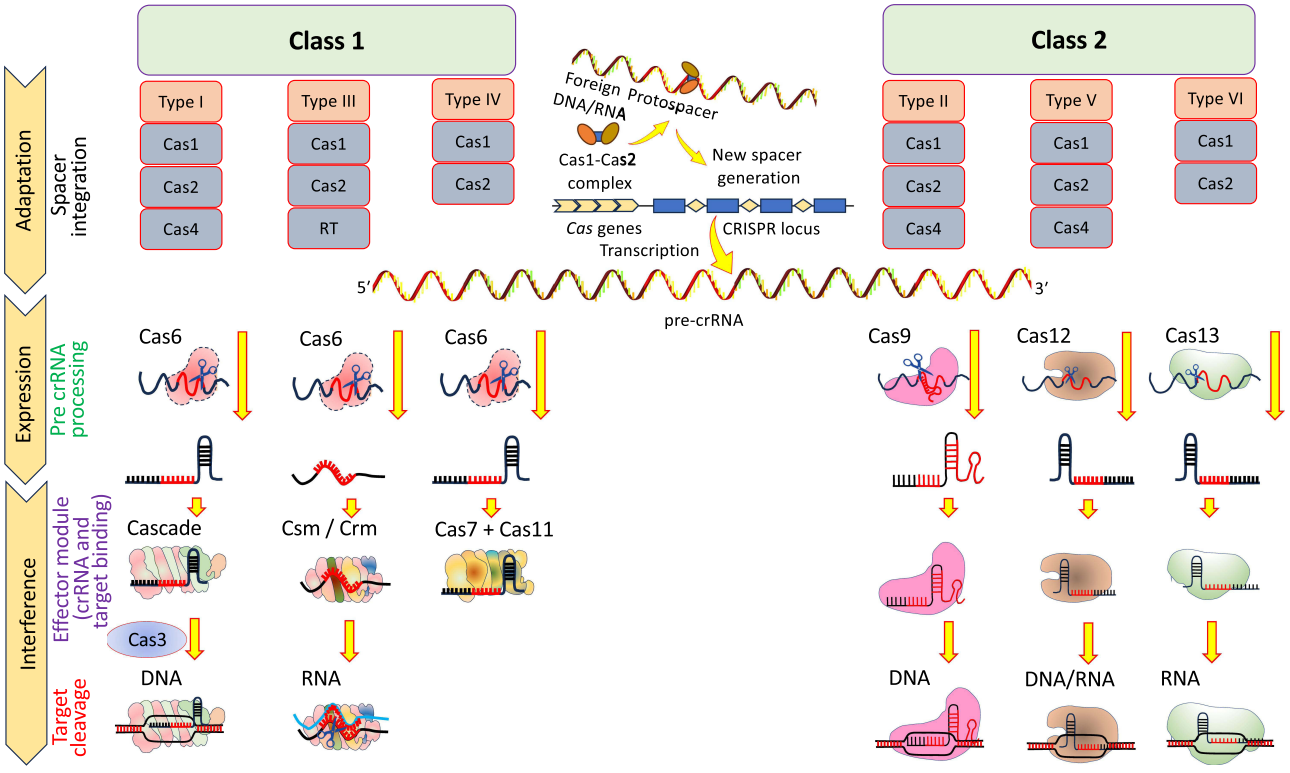

Figure 3 CRISPR/Cas system classification and functions in bacteria. Based on the generic organization, Cas effectors are classified and functional modules of CRISPR/Cas system are shown.

In this system of CRISPR/Cas classification, class 1 comprises type I, III and IV together having sixteen subtypes, incorporating effector modules composed of numerous Cas proteins. These effector modules determine crRNA and promote the pre-crRNA processing and its action. However, class 2 is composed of type II, V and VI in addition to seventeen subtypes that further consist of a single crRNA-binding protein, which is large and has multi-domain organization. It is involved in pre-crRNA processing in some variants and interference in all variants.[80,81] A unique protein composition is present in each type of CRISPR/Cas system for expression and interference steps.

Although, even with the high diversification of the CRISPR/Cas system, the primary function of this genome-editing tool remains the same. It comprises of three main steps as adaptation, expression and interference (Figures 3 and 4). The adaptation phase includes the consumption of exogenous DNA fragments into bacterial CRISPR array, followed by the expression step, which includes the maturation of CRISPR-RNA (crRNA) from the attained spacers.[82,83] The interference step involved the recognition and attachment with complementary nucleotide sequence of attacking genetic elements by crRNA, leading to its cleavage by the help of Cas nuclease[84,85] [Figure 4]. Among all different type II CRISPR/Cas systems, *S. pyogenes* derived CRISPR/Cas9 has been comprehensively studied because of its versatility, simplicity, specificity and efficiency.[75,86]

Structure of CRISPR/Cas9 System

The CRISPR/Cas9 derived from *S. pyogenes* (Spy) is the most extensively studied form of the CRISPR/Cas system as the interference step by this genome editing tool requires only single Cas9 protein (Figure 4).[87,88] The system is a ribonucleoprotein complex composed of Cas9 protein and single guide RNA (sgRNA). Cas9 protein is the DNA endonuclease enzyme (160 kDa), having 1368 amino acid residues, capable of cleaving precisely each strand of dsDNA.[89] This genome-editing tool also requires Csn2, Cas1 and Cas2 for DNA acquisition phase,[90] while pre-crRNA processing to mature sgRNA is performed by RNaseIII.[91,92]

Cas9 endonuclease consists of two lobes, the nuclease (NUC) lobe having amino acid residues from 1 to 55, and 719 to 1368 and the recognition (REC) lobe (residues 56–718).[89,93] The REC lobe performs the nucleotide recognition step by three domains

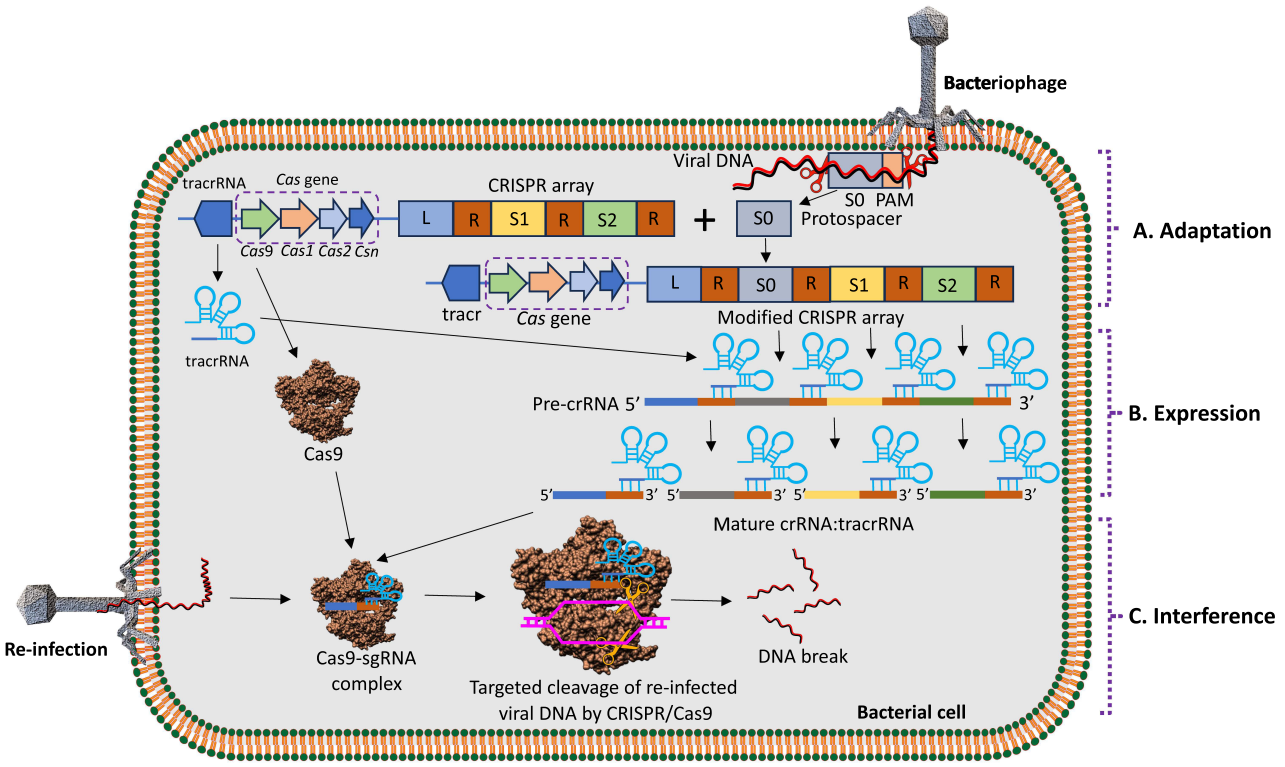

Figure 4 Mechanism of action of CRISPR/Cas9 system in bacterial cells. The three stages include, (**A**) adaptation for incorporation of foreign DNA into CRISPR loci. (**B**) During expression, CRISPR loci are transcribed and resulting pre-crRNA are further processed by Cas9/RNase III complex, generating mature crRNA which couple with tracrRNA forming sgRNA. (**C**) During interference stage, new phage re-infected DNA having any sequence matching with CRISPR spacer of sgRNA leads to cleavage by CRISPR/Cas9 complex.

named as REC (-I, -II and -III).[92,94] Cas9 contains two endonuclease domains, the HNH domain (766–909) and RuvC domain (1–55, 719–765, and 910–1099). Further, the HNH domain is responsible for target the DNA strand cleavage, whereas non-target DNA strand is cleaved by the RuvC domain (Figure 5).[93] Further, two key hinge regions as linker L1 and L2 are present in the HNH domain near N and C terminus.[95] These linkers create a crosstalk between the HNH and RuvC domains.[96] The PAM interacting (PI) domain consists of residues from 1100 to 1368 and confers the PAM specificity, responsibility for the initiation of Cas9 binding to target DNA.[89,93] With the initiation of DNA binding, the negatively charged sgRNA:DNA hybrid is stabilized by positively charged residues present at the interface between the NUC and REC lobes[97] (Figure 5). However, displaced non-target DNA is stabilized by linker region positively charged amino acid residues (L1 and L2) present between HNH and RuvC domains.[92]

The action mechanism of CRISPR/Cas9 involves different conformational changes in its three-dimensional structure as supported by X-ray crystallography studies obtained at different stages of its activity.[89,98,99] The different stages of the *S. pyogenes* CRISPR/Cas9 gene editing pathway have been resolved as free Cas9 with protein data bank (PDB 4CMQ) (www.rcsb.org),[100] sgRNA bound Cas9 (PDB 4ZT0),[99] Cas9 in association with target DNA and incomplete non-target DNA with PAM sequence (PDB 4UN3),[98] and Cas9 in association with both target DNA and complete non-target DNA (PDB 5F9R).[99] The apo Cas9 sgRNA associated Cas9 from *S. pyogenes* (PDB 4CMQ and PDB 4ZT0)[99,100] was obtained at 3.09 Å and 2.9 Å resolution, respectively. Upon sgRNA binding, a significant rearrangement occurs in the REC domain almost 65 Å shift of the REC III domain for the accommodation of sgRNA[99] (Figures 4 and 5). A further shift of the REC II domain occurs by the binding of the target DNA and PAM with an incomplete non-target DNA strand to Cas9:sgRNA complex (PDB 4UN3).[98] Melting of the foreign DNA occurs by Cas9:sgRNA-based PAM recognition and results in DNA:RNA hybrid formation.[98,101]

Mechanistic Action of CRISPR/Cas9

The mechanistic action of CRISPR/Cas9 system involves three stages as adaptation, expression and interference (Figures 3 and 4). The invading foreign DNA (almost 30 bp) is integrated into the CRISPR loci leader side during the

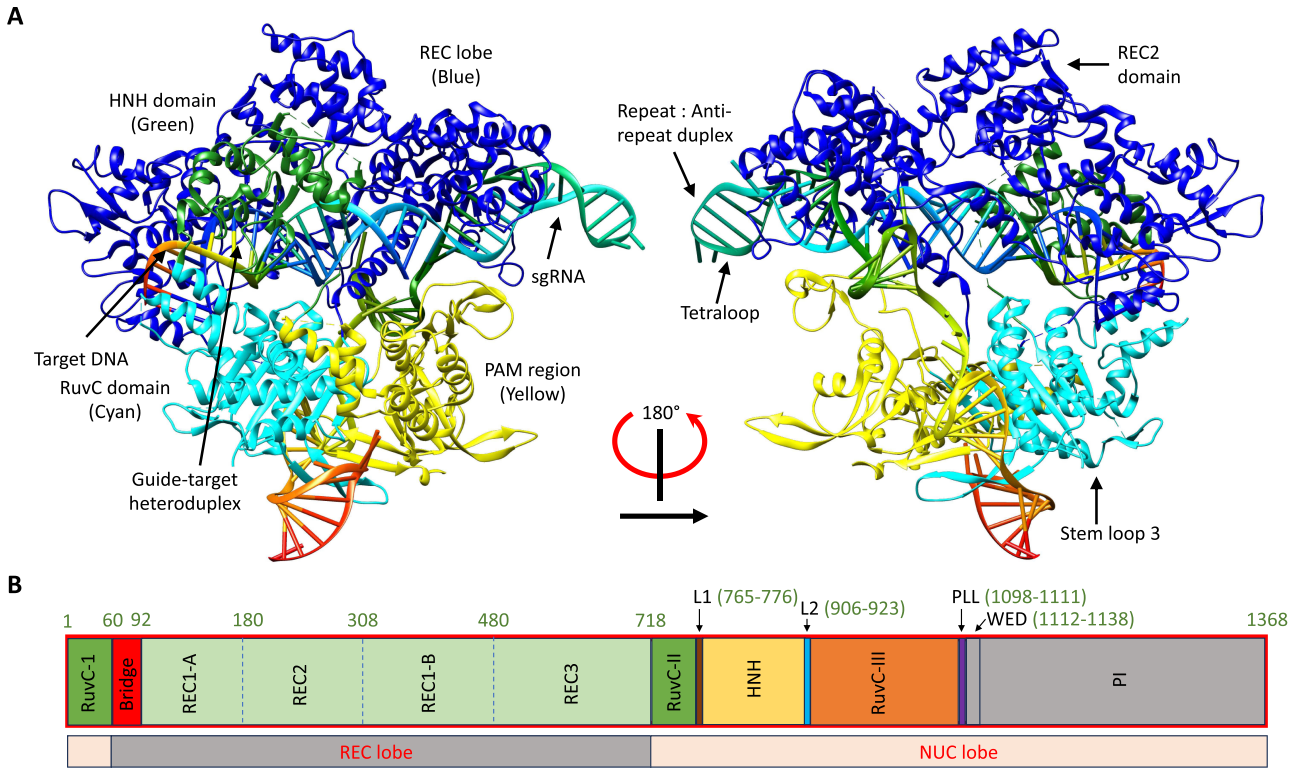

Figure 5 Three-dimensional structure of SpCas9-sgRNA-DNA ternary complex. (**A**) Ribbon representation of Cas9-sgRNA-DNA complex at two different angles, obtained from protein data bank (http://www.rcsb.org, PDB ID: 4OO8), edited by UCSF Chimera. (**B**) Domain organization representing amino acid residue numbers assigned for different protein domains.

adaptation stage. The protospacer adjacent motif (PAM) is decided from the host genome spacer sequence. The RNA transcription from spacers of the CRISPR locus occurs during the expression stage.[82,102] The discovery of using sgRNA (tracrRNA and crRNA complex) along with Cas9 has revolutionized the art of CRISPR/Cas9 system for genome engineering to new heights. In addition, as a double check, the PAM sequence is responsible for Cas9 to identify the target DNA.[103]

It has been reported from the previous study that self-targeting CRISPR/Cas system spacers assist in bacterial cell apoptosis, if the cleaved DNA repair system is delayed.[104] Consequently, it is delineated that Cas nuclease reprogramming can help in targeting precise bacterial population. This approach can repurpose the CRISPR/Cas system to kill the specific bacterial population or re-sensitize them for specific antibacterial systems.[84]

If the gRNA shows enough complementarity with the target DNA, Cas9 continues its function and generates a double stranded blunt cut, three base-pairs upstream of the PAM sequence.[105,106] As Cas9 possesses two nuclease domains, the HNH domain cleaves complementary DNA strand, while the RuvC domain cleaves non-complementary DNA strand. The DNA cleavage produces a predominantly blunt-ended double-strand break (DSB).[92,107] To repair the DNA breaks, two major repairing mechanism are used as non-homologous end joining (NHEJ) and homology-directed repair (HDR) (Figure 6). The NHEJ approach repairs the DSBs with high efficiency for gene disruptions involving deletions or insertions and occur in absence of homologous DNA sequence. However, the HDR mechanism repairs the DSBs in presence of donor DNA template with low efficiency for gene modifications.[108]

The sgRNA can be devised systematically to target virulence, MDR or other critical genes specific for pathogenesis. The CRISPR/Cas9 genome-editing system can be further engineered to induce growth inhibition of the target bacterium and its death. In addition, transcriptional repression of target genes and elimination of antibiotic resistance plasmids can be achieved by this genome-editing tool (Figure 4).[75,92]

The Novel Approaches to Combat the MDR by CRISPR/Cas9-Based Genome Editing

CRISPR/Cas9-based genome-editing is now very well considered as a promising next-generation tool to combat the infectious diseases, especially caused by AMR pathogens.[78,80] Based on the location of the target gene, CRISPR/Cas9-based genome editing

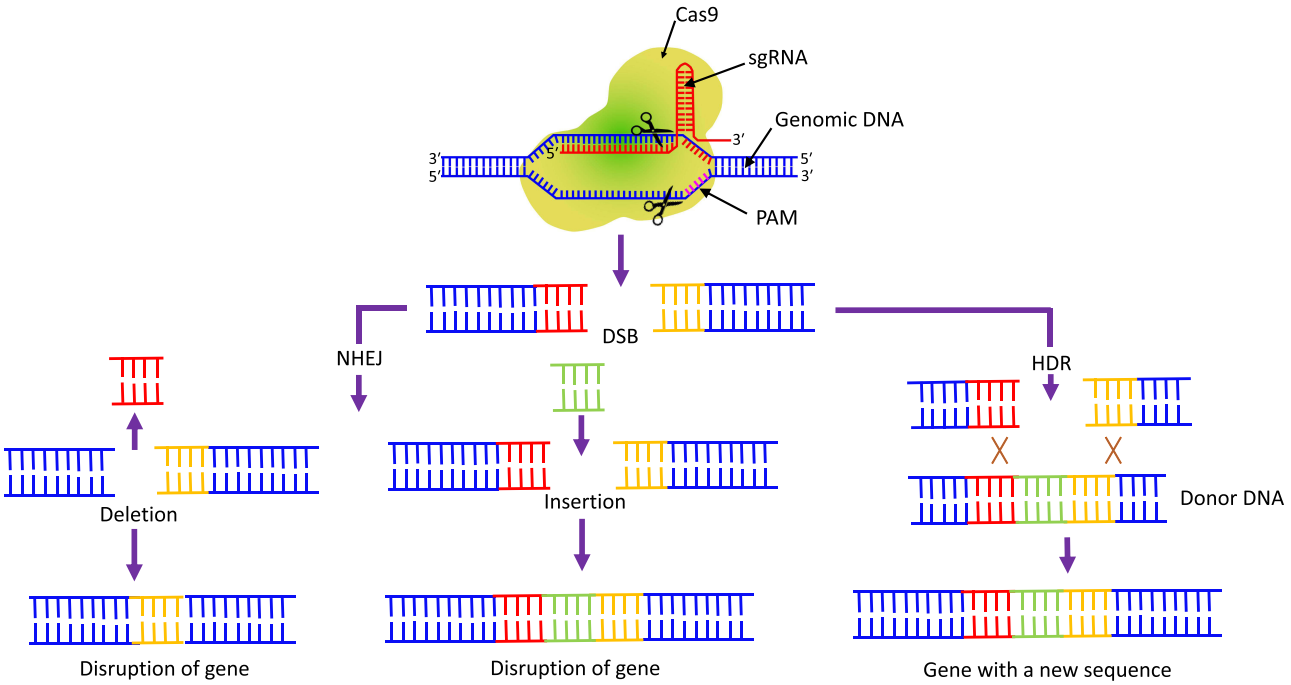

Figure 6 The CRISPR/Cas9 induced double-stranded breaks of target DNA. The breaks can joined either by NHEJ or HDR. The NHEJ-facilitated repair results in gene disruption either by the deletion or insertion of a DNA sequence. In the presence of donor DNA, HDR facilitates precise nucleotide substitutions resulting in proper gene modification.

can be utilized either as a pathogen-targeted approach or as a gene-based approach. The pathogen-based approach is relied on targeting some specific regions of bacterial chromosome. This method leads to bacterial cell destruction and killing of specific pathogenic strains. However, the gene-focused method is carried on by targeting antibiotic resistance genes carried by various plasmid. This approach leads to antibiotics sensitivity towards the bacteria.[109] Some target-specific strains of mixed microbial pathogens as well as well-defined infections can be treated by pathogen-focused approaches, whereas the gene-focused tactics are undefined. However, this approach is related to the reduction in antibiotic resistance abundance in microbial community as an approach for the treatment of bacterial infections.[110]

The CRISPR/Cas9 system is able to distinguish between pathogenic and symbiotic strains by precise sequence targeting, unlike the conventional antimicrobials. *Staphylococcus aureus* and *E. coli* have been transformed by this approach by a plasmid encoding Cas9-driven RNA. This method precisely degraded the antibiotic resistance gene expression.[110] However, this approach is currently in the preclinical phase, as the overall target is to achieve MDR therapy. However, some clinical studies by this genome-editing tool have also been performed as antimicrobial therapy. Some clinical isolates of *S. aureus* represented a decline in disease by about 50% after treatment with engineered crRNA and Cas9 for a methicillin resistance gene (*mec*A).[57,79] In parallel, another study demonstrated that this genome-editing tool targets *ermB* (erythromycin resistance gene) and after treatment, significantly minimize the advancement in intestinal erythromycin-resistant *E. faecalis*. Furthermore, CRISPR/Cas9 interventions significantly reduced the skin colonization of *S. aureus* on a mouse skin colonization model.[111]

Klebsiella pneumonia is one of the ESKAPE pathogens, which can attain multiple MDR through the mutations by horizontal gene transfer.[112] In one study, CRISPR/Cas9 genome-editing was introduced in these bacteria to study the functions of *ramR, tetA* and *mgrB* genes that mediate tigecycline and colistin resistance in carbapenem-resistant *K. pneumonia*.[113] The increased expression of CRISPR/Cas9 led to the inactivation of these genes, affecting the bacterial susceptibility to colistin and tigecycline, respectively.[113]

As MDR *E. faecalis* lacks the complete CRISPR, especially Cas9 system.[114] A proficient delivery of whole CRISPR/Cas9 genome-editing tool to these MDR bacteria by the use of pheromone responsive plasmid (PRP) was performed. This approach achieved efficient conjugation with a narrow host range limited to *E. faecalis*. A constitutively expressed CRISPR/Cas9 system was engineered with pD1, specific for the *enterococcal* antibiotic resistance genes *tetM* (encoding tetracycline resistance) and *ermB* (encoding erythromycin resistance). The erythromycin and tetracycline resistance efficiently depleted from *E. faecalis* bacteria in vitro. In a parallel context, an in vivo intestinal colonization model showed that donors having PRP targeting *ermB* potentially reduced the occurrence of erythromycin-resistant intestinal *E. faecalis*, presenting a support for the utility of engineered PRP in alleviating the multidrug resistance of *E. faecalis*.[115]

Enterococcus faecium, the Gram-positive bacteria is becoming a common cause of antibiotic resistance in hospital acquired infection. In one approach of minimizing its antibiotic resistance, high recombination rates of these bacteria by the application of CRISPR/Cas9 DNA-editing led to its targeted mutant generation of MDR clinical *E. faecium* strain E745. A deletion in the *lacL* gene (encoding a large subunit of β-galactosidase) was generated. This approach of genome editing can potentially be implemented on other Gram-positive bacteria with better recombination rates, minimizing their antibiotic resistance potential.[116]

In one study, the transfer efficiency of a conjugative plasmid TP114 containing CRISPR/Cas9 array was optimized. By the use of a single dose, this approach led to the elimination of more than 99.9% of targeted antibiotic resistant *E. coli* in mouse gut microbiota. This system was also applied to the *Citrobacter rodentium* infection model and successfully achieved the complete clearance of this infection within few days of treatment.[117]

Staphylococcus aureus is vulnerable to the powerful staphylolytic enzyme lysostaphin. It is a glycylglycine endopeptidase with significant antimicrobial activity. However, due to its wall teichoic acid, *S. aureus* shows some resistance to this endopeptidase.[118] Some researchers used CRISPR/dCas9, a variant of Cas9, to downregulate the expression of *tar*G, *tar*H, and *tar*O genes, to block the wall teichoic acid formation, and sensitizing *S. aureus* towards lysostaphin to eliminate these bacteria.[119]

In one novel study, a CRISPR/Cas9-based plasmid-curing system (pCasCure), was designed to specifically cut carbapenemase genes such as bla_{KPC}, bla_{NDM} and bla_{OXA-48} in carbapenem-resistant *Enterobacteriaceae* (CRE). The results demonstrated that pCasCure efficiently cut these genes present in different *Enterobacteriaceae* species of *E. coli, K. pneumonia, E. hormaechei*, E. *xiangfangensis*, and *S. marcescens* clinical isolates with more than 94% curing efficiency. In addition, *par*A, *rep*A, and *rep*B genes on pKpQIL plasmid were precisely cleared to prevent the plasmid-

based carbapenemase resistance gene to resensitize the effect of carbapenem antibiotic on CRE. This experiment led to the reduction of the MIC value by more than eight times.[120]

Novel Approaches of Targeting CRISPR/Cas9 System in Bacterial Cells

The targeting methods of CRISPR/Cas9 genome-editing tool to different types of bacteria confronts many challenges, since the active 160 kD ribonucleoprotein complex must pass across the bacterial cell wall to perform its activity. Several research groups have skillfully exploited species-level specificity of plasmids and phage for this genome-editing system delivery. Some novel targeting approaches also involve the nanoparticle encapsulated phage-CRISPR/Cas9 system delivery methods.[87,121] However, the search for more innovative targeting approaches is going on to minimize comprehensively the antibiotic resistance complication in the near future. A brief description of some delivery approaches of CRISPR/Cas9 system through phages, plasmids, nanoparticles and extracellular vesicles in different bacterial cells is given below.

Phage-Mediated Delivery

As pages are natural bacterial predators and efficiently inject their genomic contents within bacterial cells. Recently modified phages have been incorporated with CRISPR/Cas system to use it as a delivery vehicle.[122] The method involves the incorporation of CRISPR-DNA in plasmid constructs within a DNA-phage system, termed as CRISPR-Phage or CR-phage. Since phage makes adhesion on target bacterial cells through different receptors like pili (Gram-negative), flagellin, protein porin OmpC, peptidoglycan, teichoic acids, teichoic acid-peptidoglycan polymer (Gram-positive), and lipopolysaccharide (LPS). The adhesion between phage and pathogenic bacteria leads to the transfer of CRISPR-conjugated materials.[123,124] The exogenous Cas enzyme within the pathogenic bacteria reciprocates the genetic changes and reduces its antibiotic resistance.[79] In addition, some factors like phage pharmacodynamics, host range, and phage resistance can be modulated to enhance its effectiveness in the system.[84] Moreover, the immunodynamics and targeted gene expression in the relationship with host-pathogen can be modulated, thereby making a perfect cooperation for the effective resistance.

E. coli was transformed with a plasmid having CRISPR/Cas9 genome-editing tool against the MDR genes. In the presence of the selection agents, this approach led to an almost one-thousand-fold decrease in transformation efficiency. The novel results led to a new foundation of experiments to use phages to package CRISPR/Cas9 antibiotic resistance gene vectors. It resulted in rapid killing of specific bacteria. In a similar fashion, the virulent strain of *S. aureus* having antibiotic resistance was targeted by CRISPR/Cas9 encoded in phage.[85] This procedure also supported the species-specific targeting and killing of bacteria by this genome-editing tool. For the support of this novel procedure further, an in vivo mouse skin colonization infection model was made. The spread of the colonization of bacteria in this mouse model was significantly reduced by topically applying the CRISPR/Cas9 supported phages. Altogether, these findings reveal that properly selective pathogenic bacteria can be terminated by CRISPR/Cas9 antibacterial tools, while leaving behind the non-targeted bacteria unaffected. This approach is highly significant for the development of antibacterial approach for gut microflora and other environments.[125,126] These experiments support the practicability of CRISPR/Cas9 repurposing to attack any antibiotic resistant pathogenic bacteria rather than acting as a defending approach. This approach is expected to work efficiently in the future against newly emerging antibiotic resistant bacterial strains.

The adaptability of CRISPR/Cas9 genome editing by altering just the gRNA sequence enhances its approaches for the management of MDR. Furthermore, the engineering of phage scaffolds can also increase the delivery approaches of CRISPR/Cas9 antibacterial based on species-specific needs. Besides addressing the externally and topically treatable infections, eg, methicillin-resistant *Staphylococcus aureus* (MRSA), further approaches are required to address intracellular or tissue/organ specific bacterial infections.

Phage-CRISPR/Cas complex can address only external and surface infections, thus limiting its use for tissue specific and intracellular bacterial infections. To overcome all these limitations, CRISPR/Cas-phage complex have been encapsulated within some other nanoformulations to increase their resistance against some unfriendly conditions (eg, pH difference, antibodies etc.) and increase their availability around a specific area. These nanoformulations include liposomes,[127–129] fibers, nano-emulsions[130] and hydrogels etc.[131,132] (Figure 7).

Liposomes have been frequently used as drug delivery vectors and this approach was used to deliver phage to enhance their antibacterial properties. This approach protects the phage from neutralizing antibodies and removes the

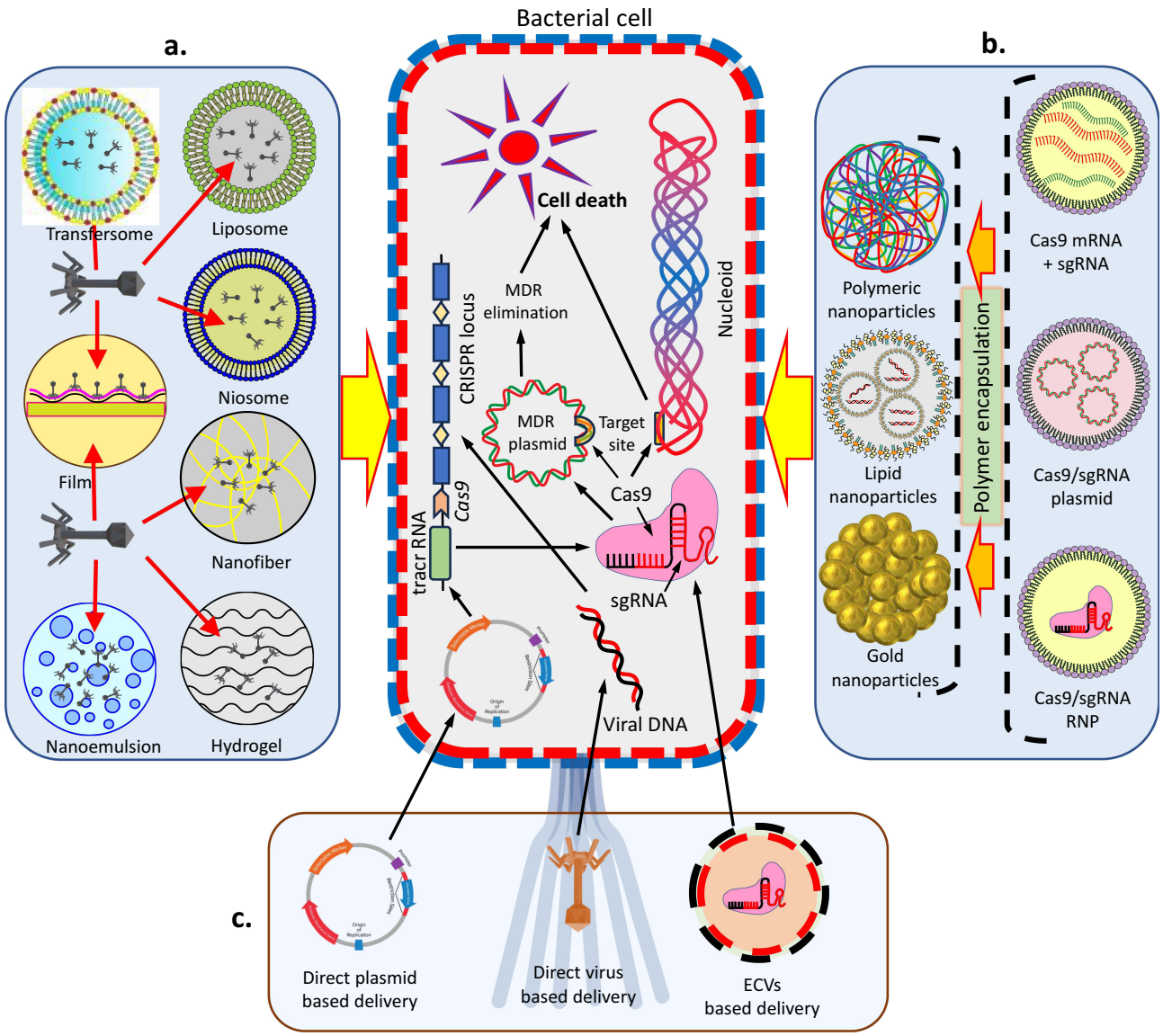

Figure 7 Different methods for the delivery of CRISPR/Cas9 genome-editing tool in MDR bacteria. a. Methods of bacteriophage encapsulation to prepare different types of nanoformulation for targeting bacteria. b. Different methods for the delivery of CRISPR/Cas9 components as mRNA, plasmids or RNPs and encapsulated as polymeric NPs, lipid NPs or gold NPs to target the MDR bacteria. c. CRISPR/Cas9 system is also directly targeted as plasmid-based delivery, viral-based delivery of extra cellular vesicles-based delivery to MDR bacteria to perform the cell death activities.

bacterial biofilm. In a parallel strategy, CRISPR/Cas9-phage complex has been delivered by using alginate hydrogel, to reduce the soft tissue infection with enhanced anti-biofilm effect over time.[132] With a suitable nanoformulation and bacteriophage encapsulation approach, the CRISPR/Cas9 system can demonstrate highly efficient against MDR activity; however, there are still many challenges to overcome to achieve the clinical transformation of this novel method for antimicrobial resistance.[133]

Conjugative Plasmids Mediated Delivery

In addition to phage-mediated delivery approaches, some other alternative delivery means can be used to target CRISPR/Cas9 system in MDR bacterial cells. The approach of using conjugative plasmids as an alternative delivery vehicle can transfer the genetic material between bacterial cells[115] (Figure 7). The *Enterococcal* antibiotic resistance has been reduced significantly by CRISPR/Cas system delivered in bacterial population through plasmids.[115] The CRISPR/Cas plasmid delivery approach has been used in carbapenem-resistant *Enterobacteriaceae* to eliminate its carbapenemase

resistance (*blaKPC* and *blaNDM*) genes. This approach also re-sensitizes the drug resistant bacteria to carbapenems with better outcomes on carbapenem-resistant *Enterobacteriaceae*.[134] The CRISPR/Cas system plasmid recipient bacteria further transmit this system to other bacteria, thus significantly expanding the application scope of using this genome editing tool to reduce the drug resistant genes.[135] The CRISPR/Cas system targeted through plasmids (TAPs) can be efficiently transferred to *E. coli* and associated Gram-negative *Enterobacteriaceae* to re-sensitize these bacteria carrying pOXA to prevent the drug resistance spread.[136]

In a similar fashion, Dong et al designed conjugative CRISPR/Cas9 against mobile *E. coli* colistin resistance gene (*mcr-1*). The CRISPR/Cas9 system re-sensitize the pathogens to antibiotics and exhibits the cells to gain immunity against *mcr-1*. The pMBLcas9-sgRNA recombinant plasmid retained the capacity to transfer into *E. coli* possessing diverse MCR-1 plasmids, significantly eliminating the MDR plasmids.[111]

In contrast to phage-based delivery, that requires a specific receptor on the bacterial cell for its attachment, the conjugation does not involve receptors for the plasmid uptake. Thus, due to the emergence of mutations in bacterial receptors, that leads to resistance against phage-mediated delivery, plasmid-based delivery is considered as a preferred procedure.[137] However, the plasmid conjugation approach also suffers from some issues, such as low delivery efficiency and narrow host range.[138]

Nanoparticle Mediated Delivery

Recently, with the rapid innovation in the field of nanotechnology, special types of nanoparticles (NPs) have been used to deliver crRNA and Cas effector molecules in the bacterial cells (Figure 7). Nanoparticles have the capacity to have flexible size and anti-degradation barrier for packaging CRISPR/Cas9 system and can maintain a natural state during gene transfer. Besides this, NPs have the advantage of biocompatibility, smaller immunogenicity, surface functionalization and higher safety as compared to virus vectors.[139,140]

Inorganic and cationic polymer-based NPs have been used to transfer the CRISPR/Cas genome-editing system components to bacterial cells.[141,142] It has been reported that a cationic polymer-based CRISPR genome-editing complex carrying the Cas9 endonuclease and crRNA can be effectively introduced into MRSA and efficiently executes the bacterial killing by targeting the methicillin-resistant gene.[66] However, the approach of delivering CRISPR/Cas genome editing system in different antibiotic resistant bacterial cells by using different NPs is still at a very preliminary stage. Many questions also need to be answered to use the nanoformulation strategy of targeting CRISPR/Cas9 system to diverse bacteria. For example, the approach of improving encapsulation rate and the achievement of efficient delivery in some peculiar pathogens such as *Mycobacterium tuberculosis*, having highly impermeable and usually thick cell wall needs to be resolved walls.[143]

In one novel study, carbon quantum dots were covalently conjugated to papG-targeted gRNA and Cas9 for the employment of Cri-dot-papG nanoformulation to deliver CRISPR/Cas system in Uropathogenic *E. coli* (UPEC). This genome editing tool targeted the papG gene (fimbrial adhesion virulence factor), thereby minimizing the pathogenicity of UPEC.[144]

Extracellular Vesicles-Based Delivery

During the growth phase, Gram-negative microorganisms synthesize outer membrane vesicles (OMVs) that move to extracellular compartment.[145,146] These vesicles can be used to deliver antibiotic resistant genes, plasmids or virulence genes as these vesicles are DNase resistant, thus serving as horizontal gene transfer methods of DNA.[147,148] Recently, these OMVs were investigated as delivery vesicles for Cas9 RNPs for gene editing purpose.[148] It was found that *E. coli* secreted OMVs can carry CRISPR/Cas9 genome-editing tools to target *Streptococcus agalactiae*, accomplishing a specific and efficient clearance of *S. agalactiae*.[149] However, the limitation of enriching RNPs within these OMVs limits the delivery efficiency[148,150] (Figure 7).

In addition to phages, plasmids, nanoparticles and membrane vesicles, electroporation procedure has also been applied to import plasmid carrying CRISPR/dCas9 into *S. aureus* and it effectively restored the clearance of Lst on these bacteria.[151] However, the complication due to cell damage and other cytotoxicity issues limit this procedure to in vitro tests only. Examples of some delivery strategies of CRISPR/Cas9-based antimicrobial genome editing tools in different types of bacteria with summarized results are listed in Table 2.

Table 2 Examples of Some Novel Delivery Methods of Designed CRISPR/Cas9-Based Antimicrobials in Different Types of Bacteria

Delivery Systems	CRISPR-Cas Locus	Bacteria	Summarized Results	References
Phage	CRISPR-Cas9	*E. coli*	Double-strand break induced by RNA-guided nucleases in bla_{NDM-1}, or bla_{SHV-18}, reducing the transduction efficiency of plasmids possessing these genes by almost 1000-fold	[152]
		S. aureus	Designed *mecA* targeting phagemid pDB91 encapsulated in phage ΦNM1 to re-sensitized MRSA to methicillin	[85]
			CRISPR-Cas9 system integration into φSaBov temperate phage genome, *nuc* gene removal from the host chromosome, host specificity expansion of phage attained by complementing the tail fiber protein	[65]
Conjugative plasmid	CRISPR-Cas9	*E. coli*	Delivery of CRISPR/Cas9 system by conjugative plasmid targeting the *mcr-1* gene, restoration of the antibiotic sensitivity to polymyxin.	[153]
			A novel approach based on TAPs by using bacterial conjugation for the delivery of CRISPR/Cas9 genome-editing tools with strain specific antimicrobial activity. The plasmid-borne carbapenem resistance gene directed by TAPs potentially re-sensitize the drug against this strain.	[154]
			Antitoxin genes (*nikA, sopA, vagC, mcr-1* and *hicB*) were used as target genes for the clearance of drug-resistant plasmids	[111]
			The CRISPR-Cas9 genome-editing system incorporated on pMob-Cas9 plasmid and conjugated to *E. coli* to target the clearance of the *mcr-1* gene	[155]
		S. algae	bla_{OXA-55}-like, *sul2*, and *NmcR*-like genes are targeted by CRISPR/Cas9, rendering *S. algae* less resistant to carbapenem antibiotics	[156]
		E. coli, Salmonella	IncP RK2 conjugative system based plasmids can be exploited as delivery vectors for TevSpCas9 dual nuclease. Non-essential genes targeted by single or multiplexed sgRNAs resulted in high *S. enterica* killing rates	[157]
		E. faecalis	CRISPR/Cas9 genome-editing tool encoded on pheromone-responsive conjugative plasmid was efficiently transferred to *E. faecalis* for the selective removal of *tetM* and *ermB* genes	[115]
Mobile genomic island	CRISPR-dCas9, CRISPR-Cas9	*S. aureus*	*S. aureus* infections is treated by highly mobile SAPIs and by the introduction of double-strand break in the *agrA* loci of chromosome, *S. aureus* is killed by ABD2003	[158]
Hydrogel	CRISPR-Cas9	*S. aureus*	The over time for Fosfomycin-phage (dual) therapeutic effects were increased by quantitative antibiofilm delivered through alginate hydrogel. The soft tissue infection was successfully reduced by this module.	[159]
Electroporation	CRISPR-dCas9	*S. aureus*	CRISPRdCas9 genome-editing tool delivered by electroporation technique in *S. aureus*, to induce the downregulation of *tarG, tarH* and *tar0* genes and to promote the bacteria to lysostaphin sensitive	[156]
Nanoparticle	CRISPR-Cas9	*S. aureus*	MRSA transfection potential was significantly enhanced by mixing sgRNA with SpCas9-bPEI to make a nano-sized CRISPR complex. This complex was designed to target *mecA*, a significant gene associated with methicillin resistance	[66]

The Challenges and Future Prospects of Mitigating MDR by CRISPR/Cas9

The CRISPR/Cas9 genome editing system holds immense potential in tackling MDR, but its application in this field still comes with a handful of challenges. Many human disease-causing bacteria such as *S. enterica, M. tuberculosis*, and *Burkholderia spp*. are well-known intracellular pathogens residing in different host cells. These bacteria offer an additional challenge to be eradicated by CRISPR/Cas9-mediated genome-editing system. Therefore, additional approaches need to be offered to transport CRISPR/Cas9 system delivery vehicles for such microbes. As the delivery of phage to eukaryotic cells by avirulent bacteria and liposomes is well established,[160–162] so it is very important to know whether these strategies could also be offered to transport CRISPR/Cas9-phage complex, conjugative plasmids and NPs to eliminate such intracellular pathogens.

Although, CRISPR/Cas9 system has been successfully used to kill bacterial cells, however, the survival of some colonies has been reported by dodging their genome targeting.[125,152] Several factors such as spontaneous mutation in spacer sequence, Cas gene or target sequence and the presence of anti-CRISPR (Acr) genes in host genome contribute to

emerged resistance against this genome-editing system.[163] Thus, to reduce this emerged resistance, future studies need to focus on preventing these spontaneous mutations in the crRNA or Cas gene.[164] Target sequence mutations responsible for widespread variants of antibiotic resistant genes, create more challenges for resistance to CRISPR/Cas9 system.

Some more challenges to implement the successful genome-editing by CRISPR/Cas9 also includes the off-target effects for in vivo therapeutic applications. Other challenges regarding the Cas9 from widely studied *Streptococcus pyogenes* (SpCas9) is its relatively large size, hard to pack in AAV vectors for subsequence bacterial genome-editing.[165]

The use of CRISPR/Cas9 system along with bioinformatics is emerging as a powerful tool to curb the problems of MDR. Bioinformatics play a significant role in designing and of sgRNA and help in decoding the bacterial genome sequence. The use of innovative computational tools and advanced algorithms in near future will help the researchers to analyze vast amount of antibiotic resistance genome information, track resistance evolution and designing of new antibiotics.

CRISPR/Cas genome-editing system display many potential advantages as compared to conventional antimicrobials. These genome-editing systems are vastly diversified, including almost 33 subtypes.[83] It is believed that more efficient CRISPR/Cas types could be exploited in the near future that could eliminate the MDR to maximum. Furthermore, the development of some more innovative CRISPR/Cas9 delivery approaches are expected in near future that will facilitate the effective genome editing in diverse bacteria to curb the MDR problems.

Conclusion

The abusive use of antibiotics in healthcare system has led to a serious impact on human lives by the emergence and wide spread of MDR organisms globally. The modern innovative approach of treating antibiotic resistant organisms by CRISPR/Cas9 genome-editing strategy proves to be a promising method to circumvent the trouble of MDR without targeting beneficial bacteria. Due to some significant features of CRISPR/Cas9, such as high specificity, versatility, efficiency, and programmability, this system can be engineered to target any MDR gene of interest to curb the problems of antibiotic resistance. However, this bacterial genome-targeting approach is still in its infantry stage and faces some challenges in its efficient targeting within bacterial cells. Some delivery approaches such as phages, plasmids and innovative nanoformulations have been engineered to target CRISPR/Cas9 to different bacterial cells, but still the research is proceeding on for better targeting approaches that kill only specific bacteria. In addition, some well-designed bioinformatics tools need to be implemented to advance the decoding of bacterial target sequences and the design of sgRNA and innovative Cas9 variants in near future to minimize further off-target binding sites. This genome-editing tool has a great potential in handling the MDR complications for clinical samples in the near future.

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