DOI: https://doi.org/10.36094/sc.v89.2024.Contours_of_CRISPR_Cas_System_to_Alleviate.Ramamurthy.445

ARTICLE

Sci. and Cult. 90 (11–12) : 445-451 (2024)

CONTOURS OF CRISPR-CAS SYSTEM TO ALLEVIATE THE ANTIMICROBIAL RESISTANCE

THANDAVARAYAN RAMAMURTHY*, GOUTAM CHOWDHURY, ASISH K. MUKHOPADHYAY, SHANTA DUTTA AND AMIT GHOSH

The improper use of antimicrobials in healthcare system and animal husbandry led to the emergence and spread of multidrug resistant (MDR) bacteria. Several approaches have been proposed to overcome the problem of antimicrobial resistance (AMR). One of the advanced approaches in controlling AMR is a clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associate protein (Cas) system. This system controls pathogen-specific AMR, without affecting the commensal bacteria. With the important features of CRISPR/Cas9, such as high specificity, flexibility, and efficiency, this system has the ability to eliminate MDR bacteria under in vitro and in vivo conditions. However, this system is still in its early stage and many challenges have to be resolved before its application in clinical practice.

Introduction

The ability of bacteria to survive and multiply in the presence of drugs is known as antimicrobial resistance (AMR). Due to improper use of antibiotics in clinical practice animal forms and aquaculture, there is a considerable surge in AMR pathogens. Such resistance can be due to a single drug or several unrelated drugs (multidrug resistance, MDR). When microorganisms become resistant to antimicrobials, regular medications become ineffective, which leads to life-threatening complications. For this reason, AMR has become a great threat to public health and notched greater awareness of the general public and the clinicians alike. With the rapid emergence of AMR pathogens, the discovery of new antibiotics seems a long-winded aspect[1]. No new class of antibiotics has been developed in the last two decades and ten antibiotics launched since 2017 are the variations of established drugs (https://www.who.int/news/item/22-06-2022-22-06-2022-lack-of-innovation-set-to-undermine-antibiotic-performance-and-health-gains. Accessed on October 15, 2024). Considering these problems, investigators concentrate their work on alternative methods for treating infectious diseases. Several strategies have been evaluated, including anti-virulence combinations, antimicrobial peptides, repurposing the drugs, live bacteriophages, vaccines, etc [2,3].

Bacteria exploit different mechanisms to acquire and spread of AMR by harboring antimicrobial resistance encoding genes (ARGs). The horizontal transfer of ARGs through mobile genetic elements (MGEs) arbitrates mostly for the spread of AMR in different species of bacteria[4]. Converting the antibiotic resistant bacteria (ARB) to susceptible ones to customary antibiotics might be a more feasible approach[5]. For this, many molecular techniques have been established. This includes homologous recombination, suicide plasmid vectors, enzyme-based transcription activator-like effector protein nuclease, zinc-finger nuclease technology, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associate protein (Cas) system, etc. These tools target specific enzymes that break double-strand DNA[6]. RNA silencing

1 ICMR-National Institute for Research in Bacterial Infections, Kolkata

* *Correspondence:* ICMR-National Institute for Research in Bacterial Infections, P-33, CIT Road, Scheme-XM, Beliaghata, Kolkata-700010, E-mail: rama1murthy@yahoo.com

and interference, enzymatically targeting the mRNA (messenger ribonucleic acid [mRNA) carries genetic information from DNA to protein via a process known as transcription and translation) with oligonucleotides and steric-blocking oligonucleotides are also demonstrated to be useful against ARB[6,7]. The CRISPR-Cas system for the targeted decolonization of MDR pathogens has also been featured in many studies[8].

This review updates information about the molecular mechanisms and modern role of CRISPR/Cas9 system in controlling the AMR.

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-CRISPR-Associate Protein (Cas) CRISPR Cas System

CRISPR protects prokaryotes from the invasion of extraneous DNA like plasmids, phage and other MGEs. CRISPR-Cas has the potential to make site-directed targeting/knockdown/reversal of particular gene. CRISPR-Cas system has two components: Cas, one of the enzymes, and a guide RNA (gRNA or sgRNA), which regulates Cas to a precise location in the genome sequence[9]. To generate the mature gRNA, the endogenous bacterial machinery processes sgRNA that is comprised of a combination of a CRISPR RNA (crRNA) and an immobile trans-activating crRNA (tracrRNA). In other words, the crRNA and tracrRNA anneal and form a gRNA (Fig. 1). Usually, Cas is flanked by partial repeats. A short DNA sequence, protospacer-adjacent motif (PAM, the NGG sequence) present at the 32 end, is important for the binding and cleaving action of the Cas. PAM is located 3-4 nucleotides downstream from the cut site of the non-complementary strand of the target DNA[9]. As shown in Fig. 1, the target DNA is the protospacer, spacer is the section of the gRNA, which anneals to the protospacer. Cas9 uncoils the dsDNA and cleave both strands upon recognition of a target sequence by the gRNA. The target DNA that contains the same sequence in the sgRNA, is a part of the external virus/plasmid, but not exists in the host bacterial genome.

Classification and Functions of CRISPR/Cas

CRISPR-Cas is categorized as Class 1 (types I, III, IV) and Class 2 (types II, V, VI) system, and both collectively have more than 30 subtypes based on the Cas protein structure and module sequence, nature of the interference complex and evolutionary relationships[9]. Class 1 has multiple Cas protein, while class 2 represents as a single multidomain CRISPR-RNA (crRNA) Cas proteins. DNA is the target molecule for types I, II, and IV, whereas type VI targets only the RNA. Types III and V degrades DNA and RNA[10,11].

Three well-defined phases in the CRISPR system include, (i) the adaptation phase implicates the acquisition of the spacer sequences (a non-coding DNA between the

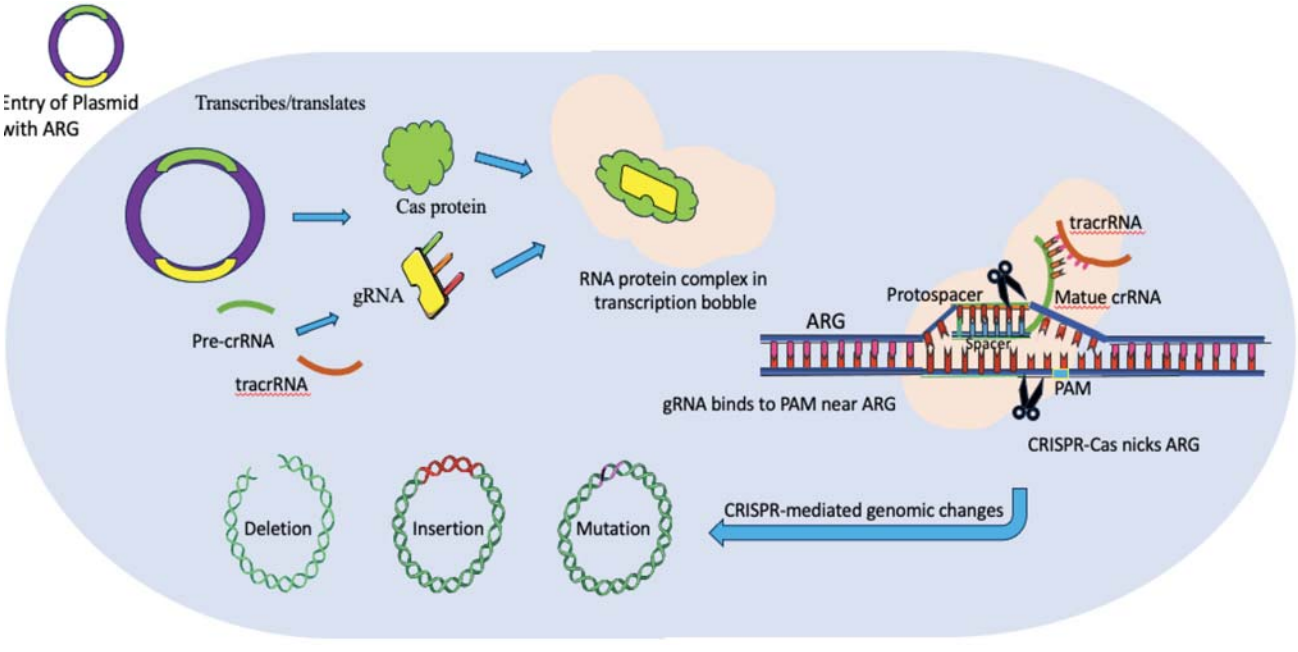

Fig. 1. Mechanism of action of CRISPR/Cas9 system in bacterial cells. The target ARG can be carried on a plasmid or/and the chromosome. The crRNA binds to tracrRNA using the base paring mechanism to from gRNA. These RNAs make a complex with the Cas9 and collectively guides to nick the dsDNA at the sequence target site coordinating with crRNA. PAM sequence helps to recognize the target DNA for Cas9 protein's binding to the target DNA. Cleavage of DNA leads to resensitization to antibiotics or cell death due to non-function of the target DNA.

genes), and integration of the invading DNA into the CRISPR locus of the host bacterial genome, (ii) the expression phase transports crRNA and the Cas proteins from the flanking spacer sequences and (iii) the interference phase includes the action of crRNA and Cas proteins that recognize the invading DNA and make dsDNA cleavage[12].

CRISPR-regulated toxin-antitoxin (CreTA) is a two-RNA toxin system that protects the diverse Class 1 CRISPR-Cas systems[13]. CreT is a bacteriostatic RNA that blocks multiplication of cells by separating a unique transfer RNA (tRNA) species, whereas, CreA is an another form of crRNA that modulates the CRISPR immune response (effector) to control (repress) *creT*. Thus, CreTA is considered as a broad anti-anti-CRISPR process that protect CRISPR-Cas against any anti-CRISPR proteins that inactivate the effector proteins, which destruct the encoding *cas* gene(s). This mechanism enhances the stability and performance of CRISPR that acts as antimicrobial.

Depending on several conditions, CRISPR-Cas system-mediated ARG regulation appears to differ. For e.g., in *Campylobacter jejuni* deletion of the *cas9* gene leads to increase in antimicrobial resistance[14]. Similarly, *Klebsiella pneumoniae* and *Legionella* spp. the AMR is comparatively high in the absence of CRISPR-Cas[15,16]. In other words, the pathogens are predisposed to gain AMR in the absence of the CRISPR/Cas.

Function of the CRISPR/Cas System

The CRISPR-Cas system has been used not only for the sensitizing the AMR pathogens, but also in the identification of pathogens, detection of ARGs and virulence encoding genes[17]. This system *per se* offer several advantages, including simple process and free from dedicated instruments. As a genome editing tool, CRISPR-Cas9 is better than the other Cas systems, as it has higher efficiency, accuracy, simpler to design to target specific genes with multiple sites without causing any complications. In addition, the CRISPR-Cas9 is an RNA-based system, which is much simpler modulate than the protein-based approaches.

Cas12, Cas13, and Cas14 are also useful in detecting bacteria and their resistance to antibiotics[18]. CRISPR-Cas13a-induced bacteria decreased by approximately three orders and demonstrated sequence-specific killing activity against extended-spectrum â-Lactamases (ESBLs)-expressed pathogenic strains[19,20]. The CRISPR-Cas13 system does not directly cleave bacterial DNA, as it targets bacterial mRNA that has lower mutation activity. Hence, the use of CRISPR-Cas13 as an antibacterial agent has greater potential. Besides, gene amplification techniques, recombinase polymerase amplification (RPA) in combination with CRISPR-Cas system is capable of amplifying the target sequence in less than 1 hour with very high precision[21]. Different delivery machineries (plasmid, phage, etc.,) help to deliver the RNA, which might take place either outside or inside the bacterial cell.

Sensitization of the ARBs

Plasmid or the other constructs with target ARG are introduced into the host AMR bacterial cells. The target can be carried on a competent vector, which leads to resensitization to antibiotics or cell death after the action of CRISR-Cas system. Inside the host bacterial cell, Cas gene gets expressed and bind to the invading DNA fragments (protospacers)[9]. After binding, the DNA sequence is inserted next to the CRISPR array's leader sequence, flanked by a repeat sequence. Following infection, the expression phase begins with the transcription of the CRISPR array and different spacer-repeat element processed into CRISPR RNA by crRNA. The Cas nuclease binds to the crRNA and this complex can then explore for corresponding protospacer, which is complementary to the spacer next to the PAM. The host bacteria cell respires the nicked DNA (double-stranded DNA that has a single-strand break) using its own non-homologous end repairing system.

To sensitize a bacterial pathogen, one or more CRISPR-Cas system has to be transformed by targeting an ARG into an *E. coli* that express the corresponding AMR. The *E. coli* expressing the ARG from a plasmid and/or chromosome can be tailored to the CRISPR-Cas system with a spacer targeting the ARG. The resulting transformants (*E. coli* that has the foreign DNA) can be allowed to grow to test sequence-specific bacterial killing by the CRISPR-Cas system. The plasmid harboring the ARG can also obliterate by the CRISPR-Cas conjugative plasmid. For this, CRISPR-Cas9 transferred to a recipient bacteria using a host-independent conjugative plasmid. This will impact the specific plasmids carrying the ARG and restrict the conjugation and transformation of AMR plasmids in bacteria. To confirm the sensitization, standard antimicrobial sensitivity assays and quantitative PCR detection of the specific ARG copy numbers are generally used. Several investigations established the function of CRISPR-Cas system in sensitizing various ARBs (Table 1).

Table 1. Sensitizing ARB using CRISPR/Cas system

Bacteria	Antimicrobial resistance phenotype	Repressed antimicrobial resistance gene	Applied CRISPR-Cas locus	Status of application	Reference
Escherichia coli	Tigecycline and colistin	*tet*(X4) and *mcr-1*	Cas9	*in vitro* and *in vivo*	35
E. coli	Cefotaxim	bla_{CTX} variants	Cas9	*in vitro*	36
Enterobacteriaceae	Carbapenem	bla_{NDM}	Cas12a	*in vitro*	37
Klebsiella pneumoniae	Oxacillin	bla_{OXA-48}	Cas9	*in vitro* and *in vivo*	38
E. coli	Cefotiam, Ampicillin	*bla*	Cas9	*in vitro*	39
E. coli	Colistin	*mcr-1*	Cas9	*in vitro*	40
E. coli	Quinolone	*gyrA*	Cas9	*in vitro* and *in vivo*	41
E. coli	Vancomycin	*vanA*	Cas9	*in vitro*	30
E. coli	imipenem	bla_{KPC}	Cas9	*in vitro*	42
E. coli	Meropenem	bla_{NDM-5}	Cas9	*in vitro*	43
E. coli	Fosfomycin	*fosA3*	Cas9	*in vitro*	44
Enterococcus faecalis	Tetracycline and erythromycin	*tet* and *ermB*	Cas9	*in vitro* and *in vivo*	45
E.. faecalis	Erythromycin	*ermB*	Cas9	*in vitro*	46
Clostridioides difficile	Erythromycin	*ermB* and *pyrE*	Cas9	*in vitro*	47
Pseudomonas aeruginosa	β-lactams, fluoroquinolones	*mex, gyrA*	1-F	*in vitro*	48
Staphylococcus aureus	Lysostaphin	*tar*	Cas9	*in vitro*	49
Mycobacterium smegmatis	Rifampicin	*arr*	Cas9	*in vitro*	50
M. smegmatis	Isoniazid	*rimI/rimJ*	Cas9	*in vitro*	51

Predictive Testing and Synergistic Action of Antimicrobials

Predictive testing explains the importance of genetic status ARGs and significant AMR in bacteria. CRISPR interference (CRISPRi) is a specific gene knockdown, which employs a deactivated or catalytically inactive Cas9 nuclease (dCas9) fused in a special repressor construct to block transcription of ARGs, without cutting the bacterial DNA. This technique has been used as a multipurpose system that prevents the ARG expression. In *K. pneumoniae*, CRISPRi system plasmid, harboring a *dcas9* gene and sgRNA regulated by the anhydrotetracycline-induced promoters has shown to repress bla_{NDM-1} and bla_{SHV-12}, with a high reduction in the meropenem and aztreonam resistance, respectively[22].

In the treatment of tuberculosis (TB) infection, long-term chemotherapy with a combination rifampicin, isoniazid, ethambutol, and pyrazinamide has been used. Withdrawal of this regime at any point may lead to the emergence of drug-resistance TB. Therefore, discovery of effective drugs to shorten the treatment period and prevent AMR is anticipated. In line with this hitch, CRISPRi has shown a collective action of cyclomarin A, a unique TB drug, with isoniazid or rifampicin[23].

CRISPR/Cas in Manipulating the ARGs

Using the CRISPR-Cas system, AMR can be controlled by (i) targeting the Cas proteins to bacterial species-specific sequences, (ii) directing the system to nick ARGs, which kill the specific bacteria carrying them, and (iii) modification or silencing the ARG maintaining plasmids to sensitize the pathogenic bacteria. In this process, most of the AMR pathogens will die or become susceptible to antimicrobials[18]. However, a small size of population might survive if there is any mutation in the target ARG. Thus, CRISPR exterminate ARB even in the presence of other beneficial bacterial population or alleviate their resistance.

As shown in Table 1, class 2 subtype II (e.g., Cas9) of CRISPR systems has been widely used in the elimination of AMR due to its simple structure that allows easy genetic manipulations. In most of the studies, plasmids carrying the carbapenem-resistance and colistin resistance

mechanisms have been focused as they are speeding across many bacterial species. Apart from ARGs, the other AMR mechanism has also been targeted. For e.g., in *Salmonella enterica* serovar Typhimurium, elimination of the *acrB* encoding acridine resistance protein B (multidrug efflux pump subunit AcrB) by CRISPR/Cas9 system has been demonstrated for the susceptibility to tigecycline[24]. Repression of the efflux system MexAB-OprM using the type I-F CRISPRi-based programmable gene silencing enhances the sensitivity of *Pseudomonas aeruginosa* to levofloxacin and amikacin[25].

Compared to the *in vitro* studies, *in vivo* testing are generally less due specific and stable CRISPR-Cas delivery mechanism. Moreover, in mouse model studies, the CRISPR-Cas machinery has shown to reduce infections caused by MDR *Staphylococcus aureus*, *Clostridioides difficile* and *Enterococcus faecalis*[26]. In *K. pneumoniae*, a native CRISPR system has cured the IncFII plasmids carrying ARGs via conjugation[27]. This method showed improved plasmid curing ability both *in vitro* and *in vivo* in a wax moth (*Galleria mellonella*) assay[27].

CRISPR Constructs to Prevent the Spread of AMR

CRISPR-Cas system has been commonly used as a genetic cargo for delivery of Cas nuclease and gRNA. Self-transmissible broad host range conjugative plasmids is a good option to deliver multiplexed CRSIPR-Cas system that can specifically target the AMR pathogens. The competence of the CRISPR-Cas system could be increased by targeting the specific genes on AMR plasmids or by generating many nicking sites on one CRISPR array or more than a gRNA[28]. A broad host-range IncP1-plasmid having the *cas9* has been designed to target ARGs with the capacity to prevent acquisition of AMR plasmids and also to delete innate plasmids from the host[29]. In staphylococci, lactobacilli or enterococci, vancomycin resistance is very common. Recipient vancomycin resistant bacteria were eliminated when the *vanA* harboring plasmid was delivered through horizontal transfer using a CRISPR-Cas9 system[30].

CRISPR system could remove several multiple plasmids carrying ARGs encoding â-lactamase. This system was effectively used in deleting $bla_{\text{NDM-1}}$, $bla_{\text{CTX-M-15}}$ harboring plasmids through phage delivery mechanism[31]. Mating assays under *in vitro* condition results in conjugative delivery of plasmids with CRISPR-Cas9 transporting multiplexed gRNAs targeted for virulence and ARGs can kill extended-spectrum cephalosporin resistant *E. coli* O157 and *Salmonella enterica*[32]. In addition, in the *in vivo* models, the conjugative CRISPR-Cas9 specifically eliminates the AMR pathogens without affecting the normal microbiota[32].

Challenges and Trajectories

CRISPR is a new process of genome modification system, which has overcome the constraints of other longstanding methods. This technology has been used for curative gene editing in health ailments like cardiovascular, muscular degeneration, neurological, optical, renal and blood disorders.

Most of the studies on CRISPR-Cas have been made using *in vitro* conditions. The existing targeting approaches of CRISPR/Cas9 delivery to bacterial cells include use of plasmids, bacteriophages, nanomaterials and vesicles. However, these approaches to specific bacterial species remains a challenging task. The other constraint realized in CRISPR/Cas9 technology is the specificity of the gRNA or crRNA that eventually describes the ability of the system. Intercellular pathogens like *Shigella, Salmonella, Mycobacterium tuberculosis* etc., instigate a big trial to CRISPR/Cas9-mediated genome-editing system. Hence, novel maneuvers has to be established to effectively deliver the CRISPR/Cas9 system.

The action between bacterial CRISPR-Cas systems and eukaryotic cells is multifaceted, which require advanced research efforts. The main challenge in validating the CRISPR-Cas-based sensitization of AMR pathogens in the *in vivo* models and ideal delivery system in a milieu that has complex bacterial population. For this reason, CRISPR-Cas is not directly beneficial to the clinical purposes. CRISPR/Cas9 cell-based gene therapy has been approved by the FDA for the treatment of sickle cell disease in children (https://www.fda.gov/news-events/press-announcements/fda-approves-first-gene-therapies-treat-patients-sickle-cell-disease Acessed on October 17, 2024). This vouches recognition and approval of other clinically promising CRISPR-based remedies, including sensitization of AMR in the future.

The potential use of CRISPR-Cas technology, extensive research and developments are in progress, especially on the delivery approaches and safety issues like removal of off-target effects, de novo resistance etc. However, the use of CRISPR technology pre-clinical animal models has not been translated into clinical use. Several preclinical studies involving the CRISPR/Cas9-mediated correction of human genetic diseases have been described[33]. Specific ethical and safety apprehensions exist

especially with the implementation of this technique. The most critical subject is the safety and adverse effects in clinical applications. Precise regulations and guidelines are required to confirm reliable use of CRISPR technology[34]. ❐

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