EXPLOITING THE BACTERIAL ADAPTIVE IMMUNITY TO DEVELOP NEW ANTISENSE – BASED STRATEGIES TO COMBAT ANTIBIOTIC RESISTANCE

Ovidiu Vrâncianu[1,2], Mariana Carmen Chifiriuc[1,2]*

[1]*University of Bucharest, Faculty of Biology, Microbiology Immunology Department*

[2]*The Research Institute of the University of Bucharest, Bucharest, Romania*

ABSTRACT

Introduction: Augmentation of the antibiotic resistance rates, both in hospitals and other environments, is one of the greatest challenges of the 21st century. Pathogenic bacteria have an enormous ability to adapt and survive in stress conditions, including in presence of antibiotics to which they develop multiple resistance mechanisms (modification/inactivation of antibiotic molecules, modification/absence/supplementing of target molecules, passive/active impermeability to antibiotics by porins/efflux pumps employment), which makes very difficult the research for finding the appropriate therapeutic approaches.

Objectives: In this review, we present the potential of CRISPR system in association with RNA interference to combat antimicrobial resistance.

Methods: A literature survey of the papers indexed in PubMed has been performed, using as key words: antibiotic resistance + CRISPR cas9, antibiotic resistance + RNA interference, CRISPR cas9 + antimicrobial strategies, RNA interference+ antimicrobial strategies, CRISPR cas9 + RNA interference.

Results: CRISPR cas9 is a form of adaptive bacterial immunity, which uses a CRISPR RNA, a transactivating RNA and one nuclease, to cleave in a specific way a target sequence. Studies conducted on several bacterial species have demonstrated that CRISPR's interference mechanism and nuclease activity can be exploited to fight antibiotic resistance.

Conclusions: The study of friendly and pathogenic bacteria has shown the important role of the CRISPR system in virulence, manifested through RNA regulatory elements. CRISPR technology provides researchers with a solid platform that can be used in advanced biomedical studies, genetic engineering and developing bacterial vaccines against mobile genetic elements and transcriptional regulation.

Keywords: antibiotic resistance, CRISPR cas 9, RNA interference, antimicrobial strategies.

REZUMAT

Introducere: Creșterea ratei de rezistență la antibiotice, atât în mediul spitalicesc, cât și în comunitate, este una dintre cele mai mari provocări ale secolului 21. Bacteriile patogene au o capacitate extremă de a se adapta și de a supraviețui în condiții de stres, inclusiv la acțiunea antibioticelor față de care pot dezvolta mecanisme de rezistență multiple (modificarea/inactivarea antibioticelor, modificarea/absența/sinteza adițională a moleculelor țintă, impermeabilitatea pasivă/activă prin intermediul porinelor/pompelor de eflux), care fac dificilă identificarea unor strategii terapeutice eficiente.

Obiective: În acest review, prezentăm potențialul utilizării sistemului CRISPR în asociere cu ARN de interferență, pentru dezvoltarea unor noi strategii de combatere a rezistenței la antibiotice.

Metode: S-a realizat un studiu al literaturii de specialitate, prin consultarea articolelor indexate în PubMed, selectate utilizând următoarele cuvinte cheie: antibiorezistență + CRISPR cas9, antibiorezistență + ARN de interferență, CRISPR cas9 + strategii antimicrobiene, ARN de interferență + strategii antimicrobiene, CRISPR cas9 + ARN de interferență.

Rezultate: CRISPR cas9 este o formă de imunitate adaptativă întâlnită la bacterii, care utilizează o moleculă de ARN CRISPR, o moleculă de ARN transactivator și o nuclează, pentru a cliva specific o secvență țintă. Studiile efectuate pe diferite specii bacteriene au demonstrat că mecanismul de interferență și activitatea de nuclează ale CRISPR pot fi exploatate pentru reducerea rezistenței la antibiotice.

Concluzii: Studiul bacteriilor comensale și patogene a arătat rolul important al sistemului CRISPR în virulență, mediat de elementele reglatoare de tip ARN. Tehnologia CRISPR oferă cercetătorilor o platformă solidă care poate fi utilizată în studii biomedicale avansate, inginerie genetică și în dezvoltarea unor vaccinuri antibacteriene direcționate față de elementele genetice și reglatorii transcripționali.

Cuvinte-cheie: antibiorezistență, CRISPR cas9, ARN de interferență, strategii antimicrobiene.

INTRODUCTION

The occurrence of multiresistant pathogenic bacteria has become a major issue at global level [1-4]. Bacteria have developed a number of specific adaptations, which allow them to escape from the inhibitory activity of conventional drugs. Survival in stress conditions is an important example of evolution and adaptation at high level due to the genetic plasticity through which bacteria

**Corresponding author:* Mariana Carmen Chifiriuc, e-mail: carmen.chifiriuc@bio.unibuc.ro

can respond to a wide range of aggressions. Bacteria use two major genetic paths to adapt to anantibiotic action: chromosomal genes mutations and acquisition of foreignDNA by horizontal gene transfer (extended spectrum beta-lactamase genes), encoding for several biochemical mechanisms of antibioresistance, consisting in the modification of antibiotic molecules (aminoglycosides modifying enzymes) or target (development of rifampicin resistance by mutations in the rpoB gene), decreasing the membrane permeability (tetracyclines resistance by efflux pumps) or subverting a certain metabolic pathway [1, 5-6].

The indirect pathogenesis concept also applies to antibiotic resistance, as normal microbiota, despite its multiple positive roles (e.g., barrier against host colonization with multi-resistant strains by competition for the same niche and nutrients, production of antimicrobial substances, stimulating the mucus layer production and the mucosal immunity) [2, 7, 11, 12, 13, 14, 15, 16]. However, the members of normal microbiota can also harbor intrinsic resistance mechanisms that could render resistant susceptible pathogens sharing the same host niche.

The great adaptability of bacteria and the increasing rates of antibiotic resistance require the rapid finding of new therapeutic approaches of infectious diseases. One of the newest and most challenging strategies that could also be applied for combating antibiotic resistance is the antisense therapy.

In bacteria, RNA elements act at transcriptional level, controlling their development or virulence, but also have an important function in antibiotic resistance. Plasmids transfer among bacteria is a major problem in the clinic, because it can lead to increased resistance to antibiotics [10].

Antisense RNA is produced starting from promoters located on the complementary strand of the target gene [8, 9]. CRISPR (Clustered, regularly interspaced, short palindromic repeats-CRISPR associated) cas is a form of adaptive immunity in prokaryotes, used to reduce the prevalence of mobile genetic elements such as bacteriophages and plasmids that can infect bacterial populations.

Therefore, the antisense RNA in combination with the CRISPR cas9 can be used to edit the bacterial genome. In this review, we present the role of RNA in antibiotic resistance, as well as the newest approaches to combating the antibioresistance phenomenon, based on CRISPR in combination with RNA interference.

Antisense RNA - structure and roles in immunity of bacteria

Antisense RNAs are single chain oligomers synthesized starting from promoters located on the complementary strand of the target gene. Antisense oligonucleotides, together with interference RNA mechanism, have an important role in blocking transcription of some genes, by complementarity based degradation of a target mRNA [17]. It has been demonstrated that these mechanisms are used to block the abnormal expression of genes involved in the occurrence of multiple diseases [18], or in the prevention of bacterial cell division [19].

In bacteria, several transcripts involved in the regulation of the many essential physiological processes have been identified. Studies on *Listeria monocytogenes* have demonstrated the existence of some transcripts with an important role in metabolism, virulence and transport mechanisms [20]. Bacteria can use these RNAs to block transcription of genes encoding for transposases, or to repress genes that cause toxin synthesis [21]. Many studies have shown that RNAs play an important role in controlling the antibioresistance phenomenon, by engaging in a series of processes, such as altering the permeability of the cell wall structure [22]. The study made by Parker and Gottesman has shown the role of small-scale RNA in regulating the AcrAB-TolC efflux system, the most important antibiotic removal mechanism found in *Escherichia coli* [23]. The action of antisense oligomers on a target gene is based on sequence specificity, and occurs in many bacterial species [24].

CRISPR cas – overview

Firstly described in 1987, CRISPR cas is a form of adaptive immunity encountered in bacteria and archaea, which has the ability to recognize „intruder" nucleic acids, and to degrade them based on sequence specificity [26, 27, 28]. The CRISPR system uses repetitive sequences of approximately 36 base pairs, separated by sequences consisting of about 30 pairs of bases (CRISPR locus) derived from

foreign DNA or mobile genetic elements [29]. This system provides immunity to similar foreign DNA through acquisition, expression and interference.

The acquisition involves engendering a spacer sequence from a short sequence of foreign DNA, and inserting it at the end of the CRISPR locus. Subsequently, this alternation of repetitive sequences and spacers is transcribed and then processed by Cas proteins, resulting in small CRISPR RNAs. The mechanism of interference demands the complementarity-binding of CRISPR RNA to foreign nucleic acid, and its cleavage by the Cas9 enzyme [27, 30] (Fig. 1).

The CRISPR system has been classified into 3 main types and 12 subtypes, each having certain structural and functional particularities. The defining feature for these types of systems is the involvement of the *cas* genes and proteins for which they encode, thus providing a wide range of biochemical functions [31]. Caspases 3 are found in all type 1 systems, type 2 is defined by the essential involvement of caspase 9, and in case of type 3, is active caspase 10 [32-34].

The CRISPR system has begun to be widely used in human genome editing in 2013. The study by Cong *et al.* [35] showed that the CRISPR system encountered in *Streptococcus pyogenes* can be reestablished to mammary

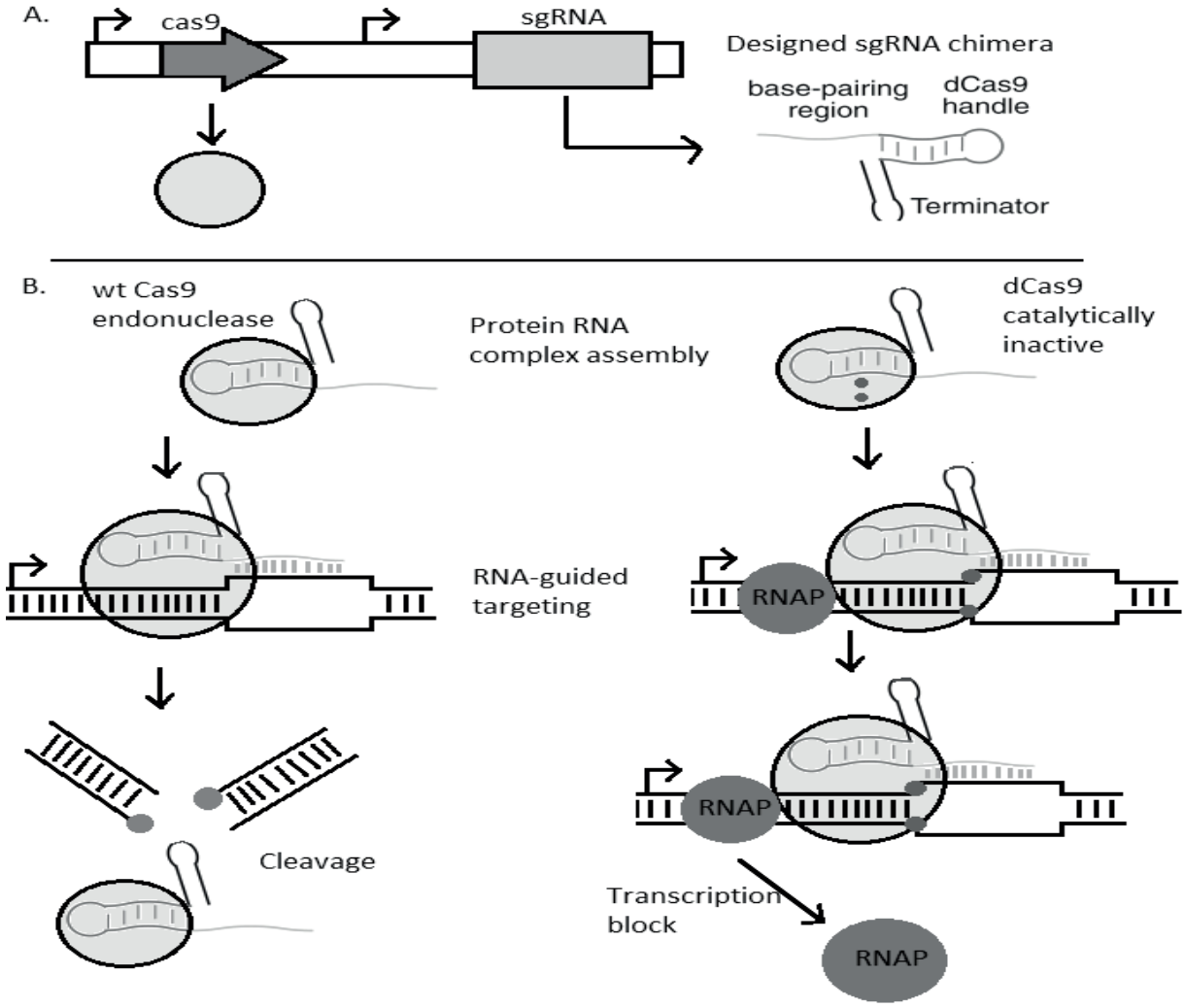

Fig. 1. CRISPR interference consists of a sgRNA chimer and a protein with nuclease activity (cas9). The sgRNA it's made of three domains: complementary region for specific DNA binding, region for Cas9 binding and a transcription terminator from *Str. pyogenes*. The wild type Cas9 protein contains the nuclease activity, in contrast with dcas9 protein. In the first stage, Cas9 protein binds to the sgRNA and forms a complex. Protein-RNA complex binds to the DNA target by complementarity between the base pairs. DNA target will be cleaved due to the nuclease activity of the Cas9 protein. When the interference acts on the protein coding region, it leads to RNA polymerase and transcript elongation blocking (modified after Qi LS *et al.*, 2013).

cells to easily manage the genome. This system consists of 4 genes, including the coding gene for caspase 9, and two non-coding RNAs, one pre-RNA and a transactivating RNA. This mechanism can be programmed to induce specific double-strand breaks in mammalian chromosomes. Subsequently, this system was transposed to other eukaryotic organisms [36-38]. Dicarlo *et al*. [39] used the CRISPR 2 system from bacteria to demonstrate the possibility of increasing homologous recombination in *Sacharomyces cerevisiae* cells. In this study, the authors used caspase 9 expressed constitutively together with a gARN cassette. Targeted double-stranded breaks may increase the homologous recombination rate in the case of two oligonucleotide donor strains.The CRISPR system is also used to specifically control gene expression in the sense of its repression or stimulation [40, 41].

The use of the CRISPR system in combination with interference RNA represents a robust therapeutic strategy to combat antibiotic resistance, either by blocking bacterial growth or by degrading virulence promoters. Both mechanisms are guided toward the target by an RNA, on the basis of which they can cleave the complementary sequence. The interference mechanism generally uses RNA up to 28 pairs of bases, and the CRISPR system uses RNA guide sequences of about 50-100 pairs of bases, including 20 base pairs which correspond to the nuclease involved in the cleavage of the target sequence [25].

Contribution of CRISPR system in combination with interference RNA for the development of novel strategies for fighting antibioresistance

Targeting multiresistant bacteria via bacteriophages introduced via the CRISPR system

Phage therapy involves their use, or protein components, to specifically target certain pathogenic strains. Genetic engineering studies have allowed phages to be manipulated to stimulate antibacterial activity. The utility of phages in the sensitization of antibiotic-resistant bacteria, by specifically targeting genes encoding the resistance property has been demonstrated [42]. Edgar *et al*. [43] performed a genetic experiment in which they manipulated the rpsL gene encoding resistance to streptomycin from *Mycobacterium tuberculosis*. Following lysogenization of the bacterium with the alpha phage encoding the rpsL gene, resistance to streptomycin was reduced. Combating antibiotic resistance through bacteriophages has also been demonstrated in studies conducted by Citorik *et al*. [44]. The authors used the CRISPR system to introduce bacteriophages into cells that specifically target certain sequences of interest, through RNA-guided nucleases (single-guided RNA). Thus, the CRISPR system can be used to remove genes encoding antibiotic resistance. Yosef *et al*. [42] have demonstrated that lysogenization with a phage leads to the sensitization of antibiotic-resistant bacteria, loss of resistance determinants, or to decrease of horizontal transfer.

CRISPR- next generation antimicrobials

Antimicrobial sequences based on antisense RNA, single-stranded oligomers with a DNA-like or RNA-like structure can bind complementarily to them RNA from a target organism. These antisense sequences bind to mRNA, and can block translation by cleaving the target [45, 46]. The specificity and design of the CRISPR system makes it possible to use it for targeting the bacterial genome [47]. For the direct recognition of the target, an important role is played by the sequence of the CRISPR spacer that binds complementarily to a target sequence of a foreign nucleic acid, called the protospacer. The protospacer sequence is framed between sequence motifs called PAM (protospacer adjacent motif), that allow the CRISPR system to differentiate self by non-self [48, 49]. The study by Hong Zhang *et al*. [50] demonstrated the possibility of genomic editing by the CRISPR mechanism, without the intervention of PAM sequences.

One of the most widely used CRISPR systems is that found in *Streptococcus pyogenes* [51], because its components can be synthesized and employed for multiple gene editing [35]. A specific CRISPR RNA, a transactivating RNA and caspase 9 nuclease are required [52]. Following hybridization of transactivating RNA - CRISPRRNA, a chimeric RNA can be generated, that can guide the nuclease to the genome (Fig. 1) [53]. Starting from the variant CRISPR encountered in *Str. pyogenes*, Citorik *et al*. [44] introduced RNA nucleases able to mediate specific cytotoxicity

into bacteriophages and in mobile plasmids, by inducing double strand breaks in some beta-lactam resistance genes [54]. It has been observed that CRISPR-associated RNA nucleases are competent to exclude antibiotic resistance plasmids and sensitize a resistant population.

Another important study is the one conducted by Bikard *et al.* [55], with the purpose of selectively kill certain multi-resistant strains of *Staphylococcus aureus*. To observe the efficiency of cleavage of certain chromosomal sequences by cas 9 nuclease, the authors constructed a plasmid by cloning the rinA, terS and terL genes into the pC194 vector. In addition to these genes, components of the *Str. pyogenes* CRISPR variant were added, i.e.: transactivating RNA, caspase 9 and CRISPR RNA, as a guide in the degradation process [52]. The plasmid was incubated with *S. aureus* cells, and the decrease of the number of viable colonies was observed. Demonstration of the ability of the CRISPR system to selectively kill resistant staphylococci was also was performed using a mice experimental model for skin colonization with kanamycin resistant *S. aureus* cells. Subsequent treatment by administering the killer plasmid at the affected area led to a decrease in the percentage of resistant colonies.

Jiang *et al*. [56] have used the CRISPR system to edit the genome in macrolide-resistant *Str. pneumoniae*. The experiment involves the integration of the ermAM gene conferring resistance to erythromycin in the srtA locus, and the introduction of a premature stop codon *via* the CRISPR mechanism, to obtain a strain with sensitivity to erythromycin. These sensitive strains can be used to quantify the ermAM gene repair efficiency. Measurement of efficiency can be achieved by transforming sensitive strains with an edited mould that restricts the wild-type allele, alongside a kanamycin-resistant CRISPR construct targeting the ermAM allele. Normally, cells receiving the CRISPR-cas9 construct are cleaved off, excepting those receiving the edited mould.The authors observed that in the absence of the mold, several kanamycin-resistant colonies can be recovered even after the CRISPR transformation. Therefore, the cells that escape the CRISPR system's action highlight the existence of some limitations of this mechanism.

Due to components that provide sequence specificity, the CRISPR system can be used to individually remove different microbial strains.To demonstrate this capability, Gomaa *et al.* [58] studied two subtypes of *E. coli* (K-12, B). Although they have a very high degree of homology [57], the difference between these two strains is represented by unique sequences found into the protospacers, near three genes (fucP, unique for K-12; ogr, unique to strain B; groL, common sequence). The authors have imagined a CRISPR system that recognizes these unique sequences as targets. The target gene fucP, led to the destruction of the K-12 strain, the ogr gene caused the removal of strain B, and groL removed both strains. The experiment was also conducted for E. *coli* K-12 and *Salmonella enterica* strains.

One of the current challenges is to find strategies to block the increase in the number of multiresistant bacteria, as a result of the transfer of plasmids that sometimes carry both virulence factors and antibiotic resistance genes. Since 2008, Marraffini and Sontheimer have demonstrated that the CRISPR-cas system has the ability to prevent the horizontal transferof antibiotic resistance among pathogenic bacteria [59, 60] (Fig. 2). Recently, Price and collaborators [61] performed an experiment in which they studied the effects of CRISPR-cas in combination with the restriction-modifying mechanism (R-M) on the conjugative plasmids transfer in *Enterococcus faecalis*. The authors used three strains of *E. faecalis*, two donor strains, OG1SSp pAM714 and OG1SSp pAM714, and an acceptor strain (T11). Subsequently, they tracked the impact of the two mechanisms on the transfer rate of pAM714 plasmid, which encodes resistance to antibiotics. Plasmid pAM714 possesses sequence motifs that will be modified by the donor strains and, following transfer to T11, the plasmid is modified and recognized as self by the T11 modifying restriction system. When both defense mechanisms are active (CRISPR-cas and R-M), the conjugation frequency is 5.4 x 10^{-6}. If the CRISPR system does not work but R-M is activated, the conjugation frequency increases to 8.7 x 10^{-4}, and when only CRISPR system is active, conjugation frequency is 1 x 10^{-3}. This experiment shows the strong impact of the CRISPR system along with the R-M mechanism on the horizontal plasmid transfer.

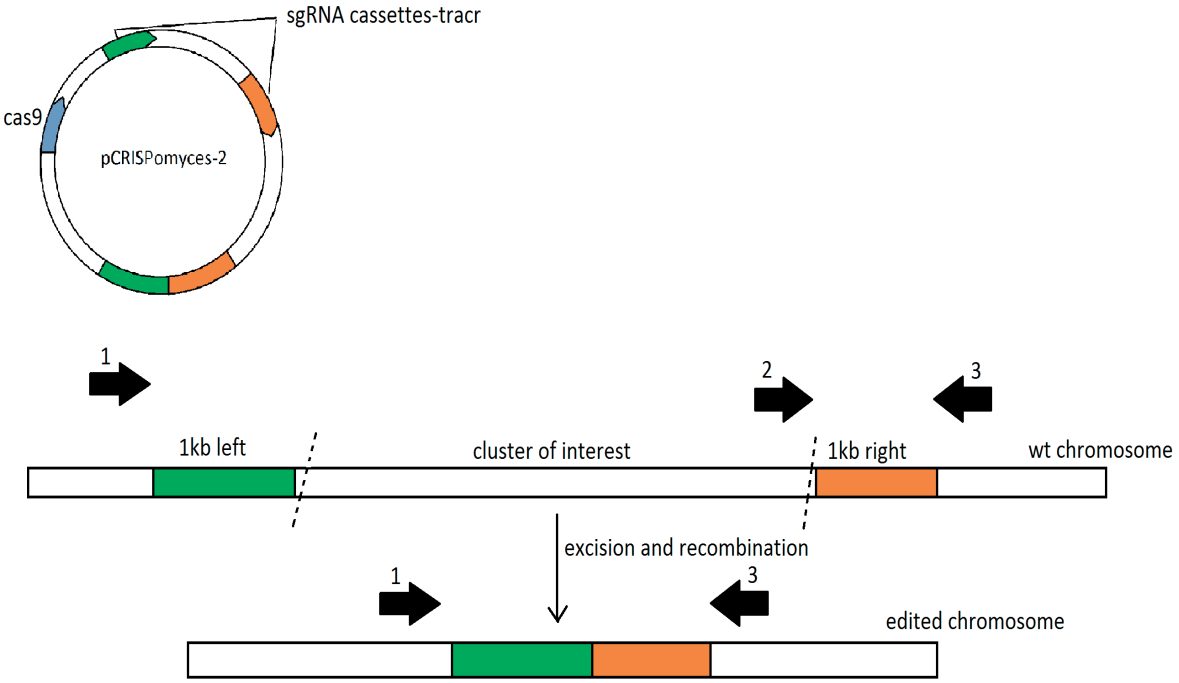

Fig. 2. pCRISPomyces plasmid able to delete a cluster of interest. This system contains two sgRNA transcripts, cas9 nuclease and other structure elements. sgRNA transcripts guide cas9 nuclease to introduce double-stranded breaks at the ends of the cluster, and an editing template bridges the gap by homologous recombination. This system can be used to edit the genome of several antibioresistant bacterial strains. For example, the caspase nuclease activity may be used to specifically target certain clusters of genes encoding antibiotic resistance (adapted after Cobb RE *et al.*, 2015).

CONCLUSIONS

The development of antibiotic resistance is a very broad phenomenon encountered in a growing number of pathogenic bacteria.

Genetic plasticity encountered in bacteria allows them to respond successfully to a wide range of aggressions, including the antibiotic attack. The study of friendly and pathogenic bacteria has shown the important role of the CRISPR system in virulence, manifested through RNA regulatory elements [62].

Antisense therapy in combating antibiotic resistance is the latest strategy involving the use of the interference mechanism in combination with the CRISPR system. Targeting RNA involved in bacterial immunity may lead to increased antibiotic efficacy following silencing genes encoding antibiotic resistance [4].

Studies conducted on several bacterial species have demonstrated that CRISPR's interference mechanism and targeted component nuclease activity allow the use of this technology to diminish the global phenomenon of antibiotic resistance. CRISPR-cas9 can attack bacteriophages and other mobile genetic elements, such as plasmids, which usually carry virulence and resistance factors. Thus, this system can act as a major barrier against the dissemination of virulence factors and the conjugative transfer phenomenon between plasmids that cause antibiotic resistance. Thus, CRISPR technology provides researchers with a solid platform that can be used in advanced biomedical studies, genetic engineering and developing bacterial vaccines against mobile genetic elements and transcriptional regulation.

Conflict of interests: None to declare.

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