Original Article

The CRISPR-Cas System: How It Works In Bacteria And How It Can Be Used To Encounter Antimicrobial Resistant Pathogens

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Abstract

The proliferation of bacteria that are resistant to antibiotic therapy presents a significant threat to public health. In lieu of the progress that has been made in antimicrobial therapy, the frequency of chronic conditions triggered by microorganisms that are resistant to such treatments is on the ascent. As a result, researchers are being forced to come up with new strategies to combat the issue. The CRISPR-Cas system, which has become increasingly popular in recent years, has been put to use to combat antimicrobial resistance in both exterior and interior pathogens. In this review, we focus on several of the application of the whole system and an option to combat the antimicrobial resistance in bacteria. We discuss a recently developed method known as a Nano-sized CRISPR system, which was used to address the genetic mutation without the assistance of a bacteriophage. The change in bacteria that are resistant to antimicrobial treatments makes it more challenging to put similar strategies into practice in the real world.

Keywords: CRISPR Cas-system, Antimicrobial Resistance, Nano-size Technology, Intracellular delivery, Encounter Bacteria, Treatment.

Introduction

Antibiotic compounds were first used approximately 90 years ago, and ever since their discovery, they had a significant impact on the field of contemporary medicine (1). Even though antibiotics have helped save countless lives by treating infections that were once fatal, the irresponsible use of antibiotics in veterinary and agricultural settings poses a significant risk because it causes an enormous quantity of antibiotics to be released into the environment. This threatens the health of humans and other organisms (2). The concurrent use of a great number of antibiotics generates enormous selective pressures, which speed up the spread and development of antimicrobial resistance genes in both

pathogenic and commensal bacteria (3). These genes for antibiotic resistance allow bacteria to evade antibiotics through a variety of strategies, such as the utilization of an efflux pump, the deactivation of antibiotic molecules by enzymes, and chemical modification (of the ribosome and the cell wall) to shield the antibiotics' cellular targets. These strategies are all made possible by the presence of these antibiotic resistance genes. When taken together, these antibiotic resistance mechanisms pose a threat to the therapeutic efficacy of antibiotics (4).

According to a report published by the Centers for Disease Control and Prevention in 2013, more than 2 million people have been infected each year with antimicrobial-resistant (AMR) pathogens, and 23,000 people lose their lives as a direct result of these infections (5). By the year 2050, it is anticipated that drug-resistant pathogens will be responsible for an additional 10 million deaths annually. This indicates that drug-resistant pathogens will become a leading cause of death, surpassing even those deaths caused by automobile accidents, diabetes, and cancer combined into a single category. In the face of adversity, bacteria can display a remarkable capacity for phenotypic and genotypic diversity. This is one reason why bacteria are becoming resistant to antibiotics (6).

There hasn't been a new class of antibiotics approved for Gram-negative bacterial infections in over 45 years, and only 37 antibiotic drugs are currently in clinical trials for phases II or III, antimicrobial resistance is primarily caused by a lack of novel antibiotic production. Research, development, screening, and testing of antibiotics all require a significant investment of time, money, and resources (7). Because of these problems, researchers felt compelled to look for alternative methods of dealing with antimicrobial resistance pathogens, and this resulted in the creation of an innovative antibacterial arsenal with enhanced target capabilities. To achieve this goal, numerous novel antibacterial peptides and nucleic acid-based therapies, bacteriocins, antibodies, and anti-virulence compounds have been developed. Bacteriophage-based therapeutics have also been developed. In this particular overview, we focus on the topic of adaptive bacterial immunity (8). The CRISPR-cas system and its possible application in the fight against the growing threat posed by antimicrobial resistance (9).

Aims of the Study

To describe in detail the molecular mechanisms of bacterial immune resistance against invading viruses using the CRISPR-cas system. To examine the possibility of using the CRISPR-cas system to battle antimicrobial-resistant bacteria, and to outline particular CRISPR-based tactics for removing such microorganisms. To emphasize the ethical problems and potential risks involved with the use of CRISPR-cas technology particularly worries about off-target effects and the possibility of unexpected consequences. Determining the present barriers to CRISPR-cas technology application, such as the need for improved delivery ways to target cells.

Development of CRISPR-cas System in Bacteria

The history of the CRISPR-cas system can be traced back to 1987 when Nakata and colleagues reported finding a set of 29 nucleotides (nt) repeats while studying the iap gene in Escherichia. coli. This event marked the beginning of the CRISPR-cas system (10). In the decade that followed, as the genomes of numerous microorganisms were sequenced, there were reports of new repeat elements emerging from the genomes of various archaeal and bacterial strains (11). In later years, the unique category of interspaced repeat sequences came to be known as "clustered repeat elements," which is now the generally accepted name for the group. Mojica and Jansen were the ones who pioneered the use of CRISPR in 2002 (12). In 2005, a significant step forward was made in this field when spacer sequences were isolated from direct repeats. These sequences had previously been linked to hypotheses regarding phage association or extrachromosomal origins. By the end of the year 2010, both the mechanism behind the CRISPR-cas system as well as its primary goal had become clear. CRISPR-associated (CAS) proteins are encoded by a cluster of genes that are located close to a genetic locus that has spacer sequences that are not repetitive. Utilizing the CRISPR-cas system for the production of phage-resistant dairy cultures for biotechnological purposes has recently begun in several research facilities (13).

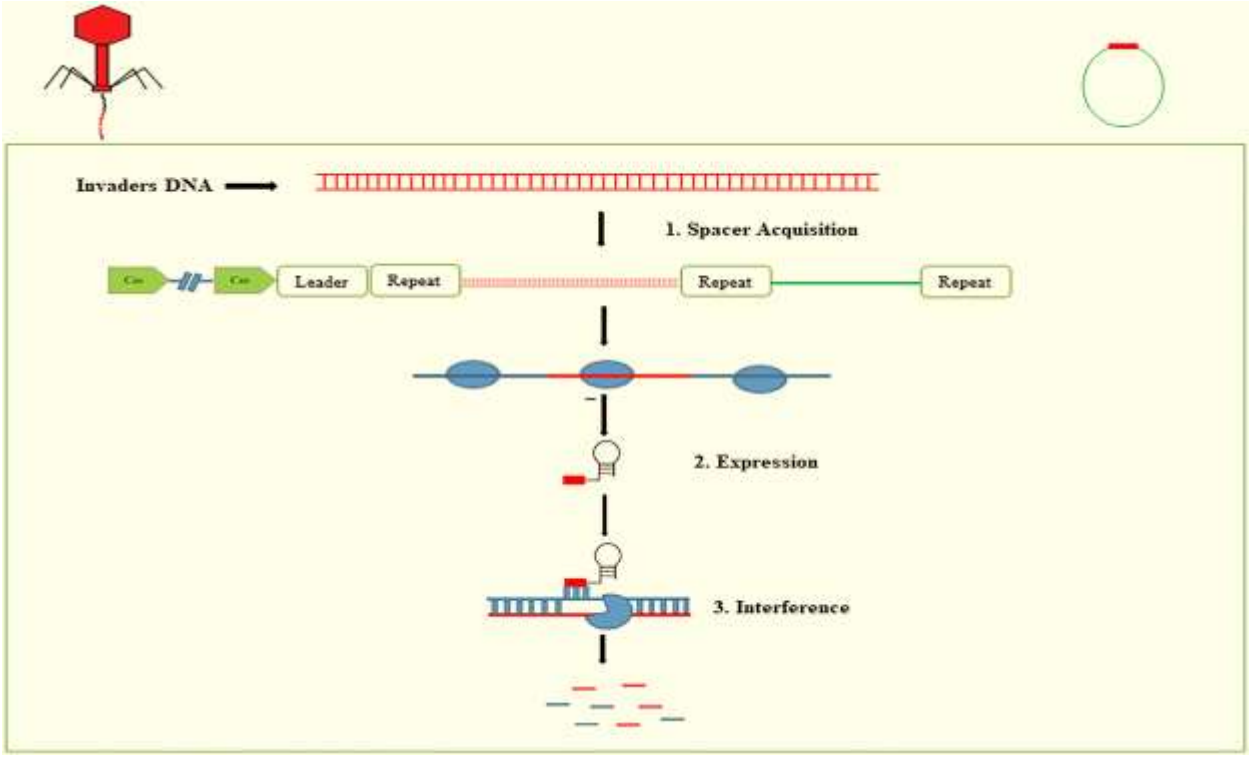

Figure 1: Mechanism of immunity against CRISPR-cas using three stages the acquisition of spacers is the initial step. At the beginning of the process, the double-stranded DNA of a virus or plasmid is inserted into the CRISPR array of the host cell at the end of the array that serves as the leader. Each set of repeats that make up a CRISPR array is broken up from one another by a unique spacer (red box) (blue box). The Cas1 and Cas2 proteins can be found in the region of the CRISPR array. These proteins are necessary for spacer acquisition and can be found there. The second stage of the process is called the biogenesis of crRNA. At this point, Pre-CRISPR RNA, also known as Pre-crRNA, is transformed into mature crRNA with the assistance of RNA polymerase located at the leader end. The third and final stage is known as assimilation, and it involves interference. The final step in the process is a perfect match between the crRNA spacer and the target sequence, which causes the rogue genetic elements to be cut apart (blue and red strips) (14-16).

The CRISPR-cas system is a component of the adaptive immune system found in bacteria and archaea. Its primary function is to defend bacteria against bacteriophages and other mobile genetic elements (MGEs) (17). The CRISPR-cas system eliminates foreign genes via a method that consists of three stages. Adaptation, also referred to as spacer acquisition, is the process that happens immediately following recognition when the spacer sequence is incorporated into the CRISPR array. Adaptation is also sometimes referred to as spacer acquisition. The second step in

the process is called biogenesis, and it is also known as the expression of CRISPR RNA (crRNA). This step requires RNA polymerase to perform the transcription of pre-CRISPR RNA (pre-crRNA) (RNAP). After that, mature crRNA is produced by cleaving this pre-crRNA with the appropriate endonucleases. Because they play a role in guiding the process, crRNAs are also referred to as guide RNAs. Interference is the final stage of the process, and it takes place when crRNAs recognize foreign RNA or DNA and form base pairs with nearly perfect complementarity that are specific to that RNA or DNA. As a direct consequence of this, the crRNA-nucleic acid complex is subsequently cleaved. The cleavage does not take place, leaving the host susceptible to infection whenever there is a DNA mismatch between the spacer and the invading organism or a mutation in the proto-spacer adjacent motif (PAM) (18-20).

Table 1: The occurrence of a variety of CRISPR-cas modules in various species of bacteria

CRISPR-cas system type-1

The vast majority of microorganisms contain CRISPR-cas systems type I (30). Such systems can be used to edit DNA. There are six distinct types of the whole system, labeled A through F, which can be characterized from one another based on variations in the cas3 gene. Cas3, which is a multi-domain protein, possesses both nuclease activity and helicase activity. The Cas3 protein can be broken down into two distinct components: the N-terminal HD phophohydrolase, which is responsible for cleaving DNA, and the C-terminal DExH helicase domain, which is responsible for unwinding double-stranded DNA. These different regions work together to remove DNA from outside sources. Cas3, on the other hand, is insufficient on its own to recognize foreign DNA and protect cells from infection. The crRNA-guided surveillance complex is an antiviral defence complex that forms in each type-1system subtype as a result of the assembly of several subtype Cas-proteins. This complex is also known as the CRISPR-associated complex (CASCADE). The discovery of and subsequent binding to the crRNA spacer-complementary target sequence is facilitated by these complexes. The crRNA-guided surveillance complex was initially described regarding E. coli K12 as it was the first organism studied (type I-E). The complex is comprised of five distinct Cas-proteins, all of which collaborate to function as a single entity. Cas6e, which was formerly known as Cse3 or CasE, now plays a role in the maturation of crRNA in its later stages. The presence of the mature crRNA, which remains attached to the CASCADE complex, contributes to the detection and cleavage of invading DNA. There have been reports of complexes in S. solfataricus that are comparable. In addition to Pseudomonas aeruginosa (type I-F), surveillance complexes that are directed by crRNA have been discovered in Bacillus halodurans (type I-C) (31), (32).

The CRISPR type-1 bacteriophage immune system is widespread among bacteria and archaea. CRISPR stands for clustered regularly interspaced short palindromic repeats. This process is characterized by the presence of multiple Cas-proteins, one of which, Cas3, is responsible for the degradation of foreign DNA. In a study that was conducted in 2015, the genomes of more than 1,000 different bacterial and archaeal species were examined. The researchers found that the type-1 CRISPR-cas system was the most prevalent of all the systems and was present in approximately fifty percent of the species. In light of this, approximately one quarter of all species have digestive systems of type-2, while only twenty percent have systems of type-2I. However, CRISPR-cas systems can be more or less widely distributed depending on the context in which they are used. According to the findings of (Marino et al., 2018) that were published in 2018, type V CRISPR-cas systems were found to be significantly more prevalent among marine

CRISPR Types	Targeted Organisms	Source
Type-1	(Escherichia coli, Pseudomonas aeruginosa, Myxococcus xanthus, Bacillus Halodurans, Campylobacter concisus, Campylobacter fetus. hominis, Campylobacter Rectus, Yersinia pestis, Salmonella specie, Erwinia amylovora, Propionibacterium acnes).	(21-24)
Type -2	(Neisseria meningitidis, Streptococcus thermophiles, Streptococcus mutans, Legionella pneumophila, Francisella novicida, Campylobacter jejuni, Streptococcus pyogenes, Mycoplasma gallisepticum, Enterococcus faecalis, Listeria monocytogenes).	(25-27)
Type-3	(Pyrococcus furiosus, Staphylococcus epidermidis, Mycobacterium tuberculosis).	(28, 29)

bacteria compared to other types of systems (33).

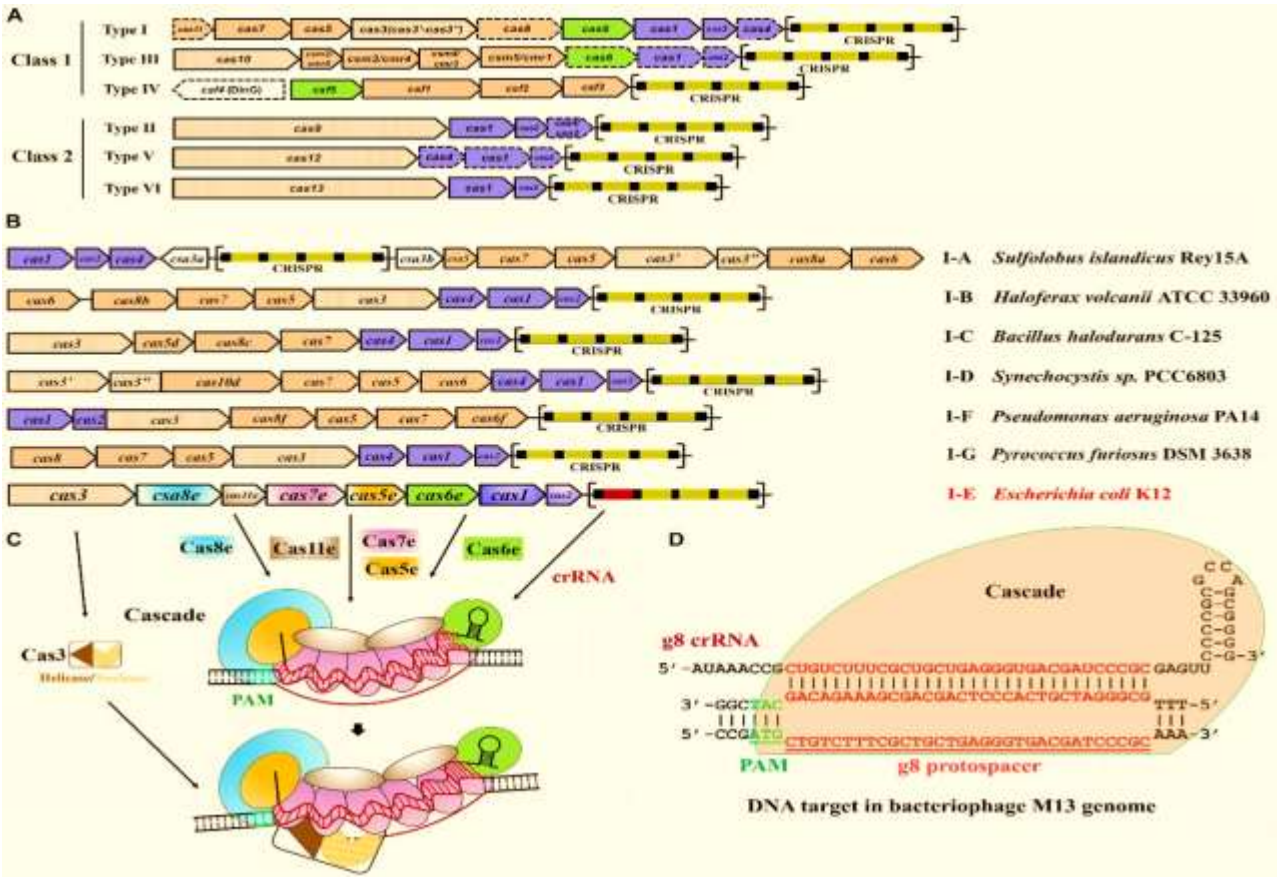

Figure 2: shows CRISPR-cas system types, architecture, and type-1 interference. Class 1 systems code effector complexes with multiple subunits, while class 2 systems code single-subunit effectors. Dotted lines indicate haplotypes without certain genes. Green and blue genes encode crRNA processing and spacer acquisition, while orange genes encode interference complex components. Four classes' effector nucleases are vertically filled bars. Squares are CRISPR repeats and spacers, while rectangles are the opposite. Common type-1subtype CRISPR-cas loci (B). Labelled genes are in all operons. Except for Escherichia coli K12 subtype I-E, gene function colours match those in (A). CRISPR arrays have square repeats and rectangle spacers. (C) Escherichia coli K12's type I-E targets DNA. Cas9 has a large (Cas8), medium (Cas9), and sometimes small (Cas10) subunit that is bound to a crRNA by Cas5, Cas6, and Cas7 subunits along the guide region (Cas11). After PAM recognition by Cas8, Cascade binds to the DNA, destabilizing the DNA duplex and allowing crRNA invasion to form a full R-loop. Cas3 is recruited to the R-loop and nicks the replaced strand of the target DNA within the protospacer. (D) The type I-E Cascade includes a crRNA (g8 crRNA) that binds to a bacteriophage M13 genome sequence (34).

The operational mechanism of CRISPR-cas type-1 system

The CRISPR-cas type-1 system can be further subdivided into subtypes I-A through I-F based on the variations in the Cas-proteins. The presence of certain Cas-proteins allows for further subdivision of these types into "modules." These modules are then further broken down into subtypes. Within the type-1 system, there is a large multi-subunit complex known as Cascade (which stands for CRISPR-associated complex for antiviral defence). Cascade is present, and it is responsible for target recognition and binding. The Cascade complex is composed of multiple Cas-proteins and a CRISPR RNA (crRNA) molecule. This molecule binds to the DNA that is being invaded and directs the Cascade complex to it. After it has become attached to the target DNA, the type-1 system will make use of the Cas3 nuclease-helicase to degrade the DNA. In addition, the Cas7 protein can be applied to cleave RNA molecules that are already

present in the system. Certainly! In microbial communities, the CRISPR-cas type-1 system is not only the most common but also one of the systems that have received the most research attention. Following its discovery in Escherichia coli, the type-1 system was discovered in a wide range of other bacterial and archaeal species (32, 35).

The elimination of foreign DNA is accomplished through a process known as cascade interference, which is specific to the type-1 immune system. The Cascade complex, also known as the CRISPR-associated complex for antiviral defence, is a group of Cas-proteins that are involved in this process. The cascade starts by attaching itself to a molecule of CRISPR RNA, also known as crRNA, and then continues to look for and attach itself to complementary sequences in the foreign DNA. Once the DNA has been attached to the Cascade complex, Cas3 is recruited to begin degrading the foreign DNA. This occurs after the DNA has been bound. In addition to being able to recognize and degrade DNA, the type-1 system also can recognize and degrade RNA. This is another distinguishing feature. A different Cas-protein called Csm6 is responsible for cleaving the complementary RNA molecules of the crRNA to achieve this goal. The fact that the type-1CRISPR-cas system is present in such a diverse range of bacterial and archaeal species is indicative of the system's significance in the context of communities composed of microorganisms (36) (37).

CRISPR-cas system type-2

Bacteria are the only other organisms that have been found to possess CRISPR Cas-system (38). The CRISPR-cas type-2 system is the most straightforward method out of all. The CRISPR-cas type-2 system is comprised of a total of four genes: cas1, cas2, cas9, and cas4 (or csn2 in the case of type-2-B). Cas9 is a protein that plays a role in both the biogenesis of crRNA as well as the cleavage of invading DNA. This is what differentiates type-2 systems from type-1 systems. The two components of cas9 known as the RuvC and HNH domains are in charge of the protein's function. The HNH domain is present in the protein to facilitate the cleavage of the strand of DNA that is complementary to the crRNA guide. On the other hand, the RuvC domain is responsible for the cleavage of the strand of DNA that is not complementary to the crRNA guide. Biogenesis of type-2 crRNA requires the presence of a trans-activating crRNA (tracrRNA) (39).

CRISPR-cas gene cluster in Streptococcus pyogenes, the coding for the tracrRNA gene takes place. During the process of crRNA repeat hybridization with tracRNA, dsRNA is formed. This dsRNA is then recognized and cleaved by the non-cas RNase III enzyme that is present in the cell. The biogenesis of crRNA is prevented when the Cas9 gene is deleted; however, its role in the biogenesis of crRNA is unknown. Jinek and his colleagues demonstrated that the cas9 enzyme is essential for the cleavage of target DNA by employing a combination of tracrRNA and crRNA. This approach was successful in demonstrating their hypothesis. Because a single protein (cas9) contains all the necessary domains for DNA cleavage, the CRISPR-cas type-2 system is extremely advantageous for genome engineering. This is due to the fact that it can cleave DNA (40).

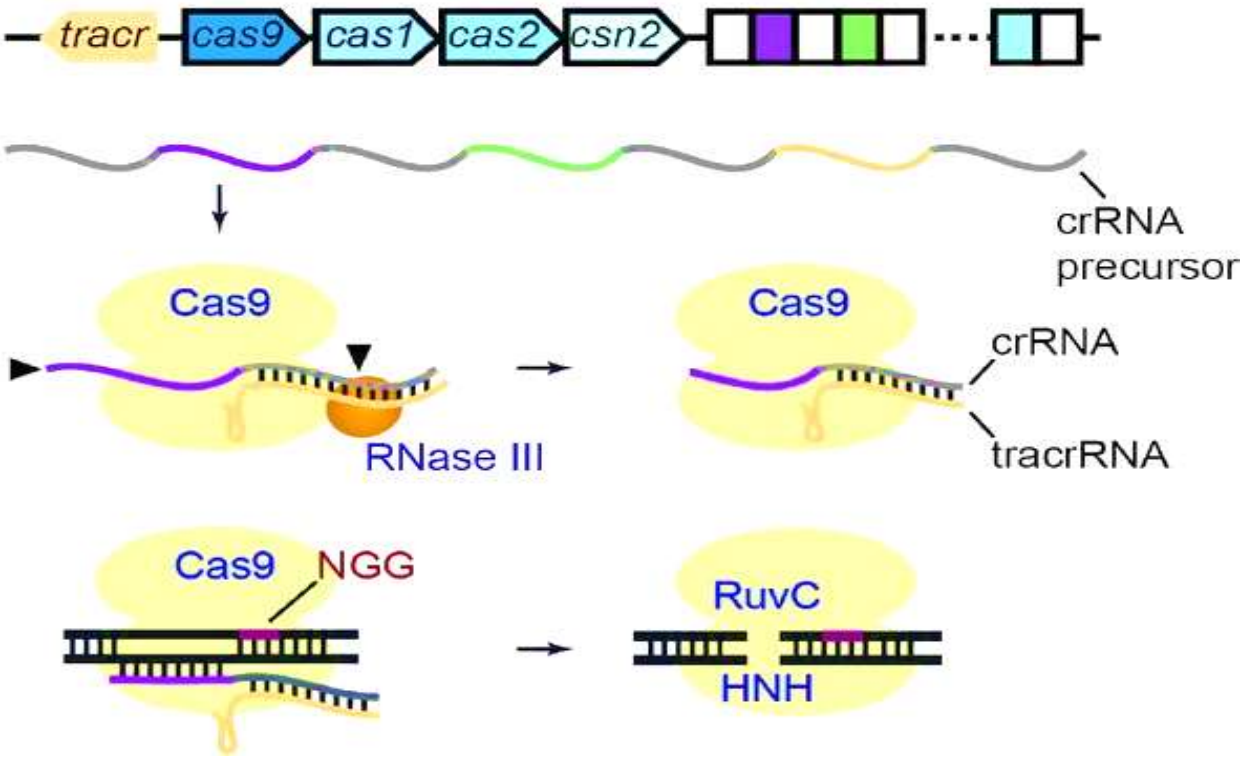

Figure 3: Working mechanism of CRISPR-cas system type-2 (the genetic organization of the type 2-A CRISPR-cas system in the Streptococcus pyogenes strain SF370. It is believed that the process of acquiring new spacer sequences involves all four genes that make up the cas operon (cas9, cas1, cas2, and csn2). The manipulation of the CRISPR RNA (crRNA). The crRNA precursor is a long transcribed RNA that contains spacers (white space) and repeats (grey line) derived from the CRISPR locus (colored lines). After the tracrRNA has interacted with each repeat sequence with the assistance of Cas9, the small crRNAs are subsequently liberated from their precursor. During this interaction, double-stranded RNA, also known as dsRNA, is produced. DsRNA is then cleaved by RNase III. The steps involved in the processing of RNA are denoted by the black arrows) (39).

CRISPR-cas system type-3

The Type 3 system can be further subdivided into two different subtypes: Type 3-A and Type 3-B. It has been reported that the type-2I-B system is only present when other CRISPR types are also present, although archaea are the organisms in which it is most frequently found (41). The CRISPR-cas type-2I system is responsible for the coding of both the cas6 and cas10 genes. Cas10, which is also known as RAMP due to its association with repeat sequences, may be involved in both the cleavage of DNA as well as the development of crRNA. Cas6 does not belong to the CASCADE complex, unlike the majority of other endoribonucleases, and it is capable of operating independently. Cascade (CAS) complexes of the type-2I system bind to and cleave foreign RNA by binding with mature crRNA. This interaction takes place during the process of the cascade (42). In addition, it is possible that archaea that use the CRISPR-cas type 1-A and 1-B systems as well as the cas6 system descended from a single ancestor. Although they are very similar, it appears that these two distinct types of type-2I CRISPR-cas systems are aimed at completely different chemical substrates. Staphylococcus epidermidis' type 3-A system is responsible for cleaving DNA, whereas the type-2I-B systems of S. solfataricus and Pyrococcus furiosus are responsible for cleaving RNA. This exemplifies the broad spectrum of CRISPR variants that can be discovered in type-2I systems (43).

Table 2: Types of CRISPR Cas systems are listed below, and they are categorized by the effector proteins they use and how they function. The effector protein for each CRISPR Cas system is Cas-3, Cas-9, and Cas-10. Effective cleavage of the target DNA necessitates the presence of a protospacer adjacent motif (PAM) sequence (5'-NGG-3', 5'-NGG-3' and 5'-TTN-3'). Guide RNAs can be designed for precise genome editing using the target sequences (5'-TAGG-3', 5'-NGG-3' and 5'-TTC-3'), which are system-specific for CRISPR Cas systems.

CRISPR Cas Type	Effector Protein	Pathogen Targeted	Target Sequence	PAM Sequence	Mechanism of Action	Source
Type-1	Cas-3	Salmonella enterica	5'-TAGG-3'	5'-NGG-3'	Cleavage of target DNA	(39, 44)
Type-2	Cas-9	Streptococcus pyogenes	5'-NGG-3'	5'-NGG-3'	Cleavage of target DNA	
Type-3	Cas-10	Pseudomonas aeruginosa	5'-TTC-3'	5'-TTN-3'	Degradation of target RNA	

The CRISPR-cas system and its function in the virulence of bacteria

It has been demonstrated through a variety of studies that the CRISPR-cas system serves purposes other than protecting bacteria from harmful pathogens. CRISPR-cas system not only regulates the pathogenicity of bacteria, but it also controls the transcription that occurs within the cell (45). Francisella novicida, which has the potential to cause disease in humans, replicates intracellularly, which provides it with protection from the immune system of the host. This bacterium will use a variety of strategies to avoid being destroyed by the macrophages that are a part of the host immune system. After being ingested by macrophages, Fusarium novicida travels through the cytoplasm to the phagosome, which is a compartment densely populated with immune recognition receptors and antimicrobial molecules (46). Toll-like receptor-2, also known as Toll-like receptor, is a type of receptor that is capable of recognizing bacterial lipoproteins (BLPs). When Toll-like receptor 2 is activated, which causes a pro-inflammatory response and recruits and activates immune cells, the bacterial pathogen that was causing the problem is eliminated. In Fusarium novicida, the expression of BLP is suppressed by regulators such as cas9, sacRNA (small, CRISPR associated RNA), and tracrRNA. Because the pathogen is able to avoid activating TLR2, it can continue to thrive inside the host even though it should be eliminated. It has been shown that deletion mutants of Fusarium novicida for cas9, sacRNA, and tracrRNA elicit a significantly more intense inflammatory reaction than the wild type strain. The fact that these mutants were unable to infect mice is a piece of evidence that lends support to the notion that the CRISPR-cas system plays a role in Fusarium novicida as a virulence regulator (47, 48).

Cas9 is connected to Neisseria meningitidis ability to attach itself to host cells and replicate on the inside (49). There is evidence that Cas9 plays a role in the attachment and invasion of Campylobacter jejuni into epithelial cells. Our working hypothesis is that the CRISPR-cas system not only assists in the attachment of Campylobacter jejuni to host cells but also protects it from the innate complement of the host cells. It has been discovered that deleting the cas9 gene from cst-II positive Campylobacter jejuni causes the organism to lose almost all of its virulence. It is hypothesized, however, that cas9 does not act alone to regulate virulence in these microorganisms. CRISPR-cas9 is a gene that plays a role in the regulation of several genes that are associated with virulence in Campylobacter jejuni. It was just recently discovered that this gene can increase Campylobacter jejuni virulence (50, 51).

CRISPR-cas system involvement in antimicrobial resistance

Multiple pieces of research have established a connection between the CRISPR-cas system and the evolution of bacteria that are resistant to antibiotics (3). Controlling BLP helps Fusarium novicida maintain the integrity of its envelope. This ultimately leads to the development of antibiotic resistance as well as resistance to other membrane stressors (52). According to the findings of (Vega et al., 2019) study, the competent strains of Aggregatibacter

actinomycetemcomitans possess CRISPR-cas systems, whereas non-competent strains of the bacterium have lost their CRISPR immune system. The results demonstrated that the development of CRISPRs and competence systems encourages the emergence of novel bacterial species as well as increases the genetic diversity that already exists. Similarly, Levin et al. hypothesized that bacteria that were equipped with CRISPR might develop resistance, which would result in a population of bacteria that had higher fitness than other variants (53).

According to the findings of a plethora of studies, the CRISPR system helps maintain genetic equilibrium by defending the host genome against foreign invaders. This is an important function that should not be overlooked. Conjugative elements, such as plasmids, can transfer genes to bacteria, which then allows the bacteria to improve their fitness in the environment by either becoming more resistant to antibiotics or becoming more virulent. The CRISPR-cas system has been shown to have a corroborating effect on the presence of plasmids and phages in several species of Enterococcus, Campylobacter, and many groups of A Streptococcus. These findings have been demonstrated. According to the findings of a single study, the application of the CRISPR-cas system to target the plasmid had the opposite of the desired effect on the antibiotic resistance of Staphylococcus epidermidis(27, 54).

Why CRISPR-cas system used to encounter antibiotic resistance threats?

Genome editing has been optimized with a variety of engineered meganucleases, including those derived from microbial MGEs, RNA-guided DNA endonucleases (cas9) derived from the CRISPR-cas type-2 system of bacteria (9), zinc finger nucleases (ZFNs) derived from eukaryotic transcription factors (3), and Xanthomonas-derived transcription activator-like effectors (TALEs) (55). Meganucleases are not typically utilized for genome editing because of the lack of sequence specificity that they exhibit for the DNA that they target. It is difficult to design ZFNs so that they can bind to a particular sequence, these tools also have some limitations. Even more so, ZFNs have fewer choices available when it comes to selecting a target site. TALENs are easy to design because they can have longer DNA-binding protein domains. As a result, they are capable of achieving a high level of targeting specificity. However, TALENs are significantly larger than ZFNs, which makes the process of delivering them into cells more difficult (56).

With the assistance of a guide RNA, the Cas9 nuclease of the CRISPR-cas type-2 system can recognize the DNA of its target by employing the Watson-Crick base pairing method. Because the sequences contained in CRISPR guide RNAs are specific to an invader sequence, it is simple for us to replace these sequences with our own to retarget the CRISPR-cas9 nuclease. The CRISPR-cas system has been the subject of extensive research, and with its capabilities, it is now possible to insert, delete, or mutate genes in virtually any species. Even the treatment of genetic disorders in living organisms has been attempted with the help of this system. Similarly, this system is being incorporated into individualized antibacterial preparations that can selectively target AMR pathogens within complex bacterial populations, target and treat only pathogenic bacteria, and in some cases treat infected host cells. The CRISPR-cas system can differentiate between bacteria that are helpful and bacteria that are harmful thanks to the sequence-specific targeting it employs. The CRISPR-cas system is discussed in this article for its potential application in the fight against AMR pathogens (57, 58).

Intracellular delivery of CRISPR-cas9 antibacterials

CRISPR-Cas9, a tool for editing genes, has experienced a meteoric rise in popularity over the past few years due to the revolutionary potential it possesses in a wide variety of fields, including medicine. One of the most fascinating applications of the CRISPR-Cas9 technology is the development of antibiotics that can target and eradicate only disease-causing bacteria (59).

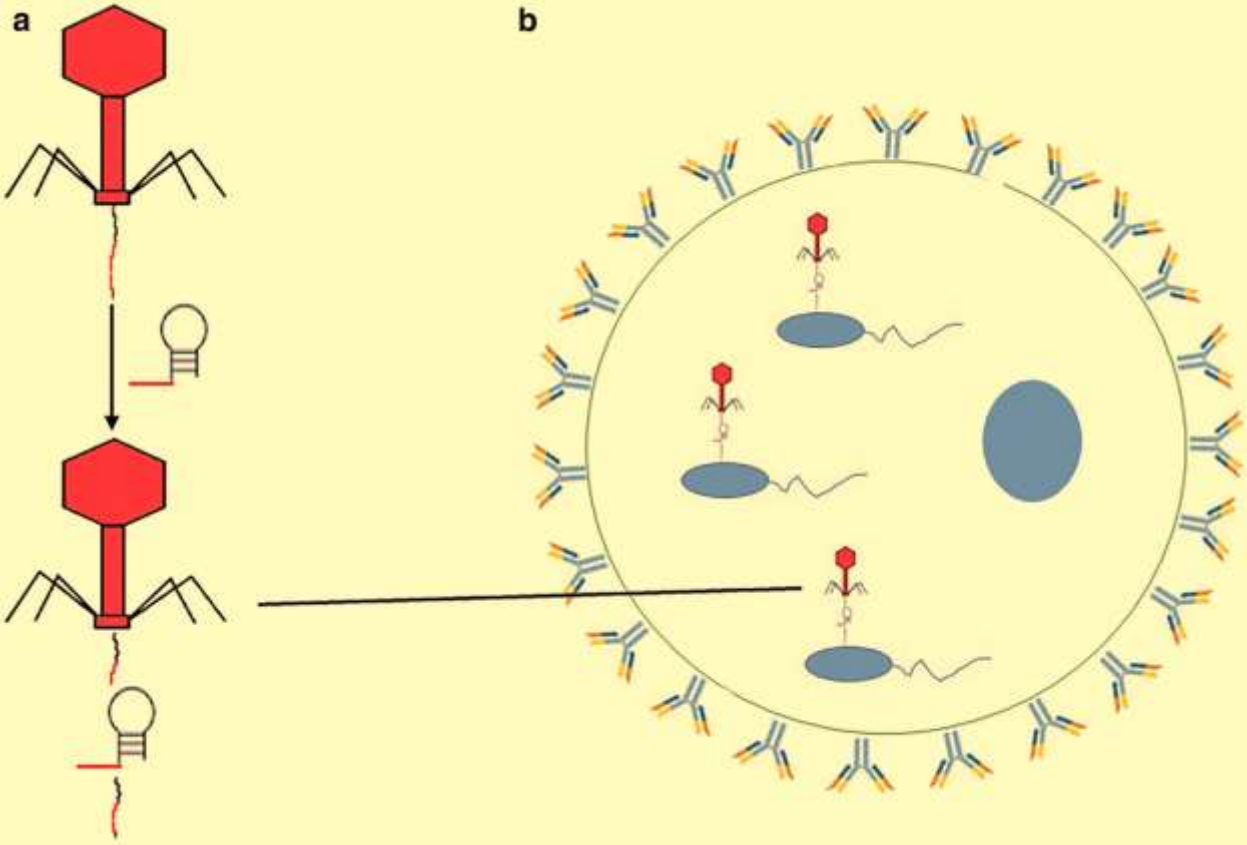

Figure 4: Transfer of CRISPR-cas9 antibacterials to a bacterial infection. (a) One of the bacteriophage-encoded antimicrobials that uses the CRISPR-cas9 system. (b) To fight AMR pathogens, phages encoding CRISPR-cas9 were introduced into infected host cells (60).

Researchers have developed a wide variety of intracellular delivery methods for CRISPR-Cas9, ranging from physical methods such as electroporation and microinjection to chemical methods such as liposomes and nanoparticles. All of these intracellular delivery methods are capable of successfully transporting the protein into bacterial cells. Transient pores in the cell membrane can be generated by exposing bacteria to an electric field. This opens the door for CRISPR-Cas9 to enter the cell and edit its DNA. To inject CRISPR-Cas9 into a bacterial cell, a micropipette is typically used. Because both of these methods require specialized equipment and personnel who have received training, it is possible that they are not well-suited for widespread application (61, 62).

It is possible to deliver CRISPR-Cas9 inside cells on a larger scale using chemical methods such as liposomes and nanoparticles. Liposomes are very small vesicles that are made up of a lipid bilayer and can package CRISPR-Cas9 so that it can be introduced into bacterial cells. However, nanoparticles, which can range in size anywhere from one to one hundred nanometers, can be engineered to carry CRISPR-Cas9 and eradicate bacteria in a targeted manner. The field of CRISPR-Cas9 antibacterials delivered inside cells is rapidly developing, with new and improved methods being tested and implemented all the time (63).

In addition to the methods, researchers are also looking into the possibility of employing phage vectors as a means of transporting CRISPR-Cas9 antibiotics (64). It is possible to modify bacteriophages so that they carry CRISPR-Cas9 and then use those phages to transport the gene-editing tool into bacterial cells. This approach has the potential to deliver CRISPR-Cas9 to pathogenic bacteria in a more targeted and specific manner, thereby reducing the amount of collateral damage caused to beneficial bacteria. Because of a phenomenon known as off-target effects, the CRISPR-Cas9 system may also target and edit genes that are not supposed to be modified. Yet another challenge for the

intracellular delivery of CRISPR-Cas9 antibacterials. Researchers are currently working on solutions to the problem, some of which include the use of modified Cas9 proteins or the development of methods to identify and eliminate off-target effects, both of which increase the specificity of CRISPR-Cas9. CRISPR-Cas9 antibacterials can be delivered inside cells, there is a lot of room for optimism regarding the development of novel and specifically targeted treatments for bacterial infections. Nevertheless, additional research is necessary to improve the CRISPR-Cas9 system's safety and specificity, as well as to identify the most effective methods of delivery (65, 74).

CRISPR Cas system in Detection of Pathogenic Gram-Negative Bacteria and Antimicrobial Resistant in Gram-Negative Bacteria

Utilizing CRISPR-Cas9, research has been done on both pathogenic gram-negative bacteria as well as gram-negative antimicrobial resistance (AMR) (66). This technique, also called CRISPR-based diagnostics, makes use of CRISPR-Cas9 to recognize and snip off DNA sequences that are unique to a particular pathogen or antibiotic resistance gene. SHERLOCK, which stands for "Specific High-sensitivity Enzymatic Reporter unlocking," is a diagnostic method for identifying gram-negative bacteria that is based on CRISPR and uses CRISPR-Cas13a to identify specific RNA sequences within the cells of gram-negative bacteria. By designing guide RNAs that target RNA sequences that are specific to a given pathogen or antibiotic resistance gene, researchers can use SHERLOCK to detect and quantify the presence of targets in bacterial samples (67, 68).

Another diagnostic method based on CRISPR is called DETECTOR (DNA Endonuclease Targeted CRISPR Trans Reporter) (69). This method uses CRISPR-Cas12a to locate and cleave specific DNA sequences that are associated with pathogens or antibiotic resistance genes. The fact that DETECTOR, like SHERLOCK, can be programmed to detect multiple targets at the same time makes it a potentially useful tool for diagnosing infections and determining the presence of antibiotic-resistant bacteria. Diagnostic tests that are based on CRISPR show a great deal of promise for enhancing our capacity to diagnose and treat bacterial infections. This will be accomplished by enabling the rapid and accurate detection of pathogenic gram-negative bacteria as well as antibiotic resistance in gram-negative bacteria (70).

One strategy involves the use of biosensors that are based on the genome editing tool CRISPR-Cas9. These devices can identify pathogenic bacteria as well as antibiotic-resistance genes in patient samples. These biosensors typically make use of a Cas9 protein and a reporter molecule that generates a signal in response to the cleavage activity of Cas9 to detect the presence of pathogenic bacteria or antibiotic-resistance genes. With the help of the biosensor's output signal, it is possible to determine whether or not a given sample contains the bacteria of interest or the resistance genes (71).

The technology known as CRISPR-Cas9 offers an additional method that can be used to determine in a short amount of time which Gram-negative bacteria have developed resistance to antibiotics. CRISPR-Cas9 is utilized in this technique as opposed to first cultivating the bacteria to achieve a more direct and productive method of identifying antibiotic resistance genes in the genomes of bacterial species. The data that is provided by this method, which is both faster and more sensitive than other methods that have been used in the past to test antibiotic resistance, can be of great use to clinicians (72).

CRISPR-Cas9 has been applied to the development of novel methods for engineering bacteria to produce antimicrobial peptides. Peptides are short chains of proteins that can either destroy pathogenic bacteria or stop their growth. Using CRISPR-Cas9, researchers can genetically modify bacteria to produce novel antimicrobial peptides, which can then be used to combat Gram-negative bacteria. The CRISPR-Cas9 technology has a significant amount of promise in terms of its applications to the diagnosis and treatment of bacterial infections, in particular those infections that are brought on by pathogenic Gram-negative bacteria and those that have developed resistance to conventional antibiotics (45, 76).

Conclusion and future prospective

With the advent of the CRISPR-cas system, researchers have a newfound appreciation for the ingenuity with which bacteria devise resistance against antimicrobials. To better manipulate bacterial genomes, scientists have developed new tools and procedures as they gain a deeper understanding of the system's basic principles. When applied to the problem of antimicrobial resistance, CRISPR-cas technology has the potential to be a game-changer.

Scientists potentially render bugs more vulnerable to antimicrobial drugs and possibly eradicate them by editing their genomes with the CRISPR-cas system. The increasing spread of antibiotic resistance has significant consequences for public health because it endangers our ability to effectively treat infectious infections. The CRISPR-cas system has promising applications beyond just combating bacterial infections. It may also be used to address genetic abnormalities and other diseases. Several obstacles must be solved as this technology advances, including concerns about its safety, effectiveness, and ethical implications. To guarantee the benefits of CRISPR-cas technology are realized responsibly and ethically, scientists, governments, and the general public will need to work together.

More and more precise approaches for targeting and modifying bacterial genomes are anticipated to emerge in the future as CRISPR-cas technology continues to develop. There is hope that these developments will help us fight antibiotic resistance and other infectious diseases more successfully. To make sure this technology is used properly and responsibly for the benefit of all people, it will be essential to keep a close eye on it and be cognizant of the risks and unintended consequences it may pose.

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