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Harnessing CRISPR-Based Technologies Against Antibiotic Resistance in Multidrug-Resistant Bacteria

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Abstract:

Due to antibiotic abuse, multidrug resistance (MDR) has developed quickly, creating a worldwide health disaster with significant morbidity and death. MDR is attained by bacteria by a variety of strategies, including improved efflux mechanisms, target protection, modification, or bypass, and antibiotic modification or degradation. Since the traditional methods of combating MDR bacteria are costly and time-consuming, it is crucial to understand the molecular processes behind this resistance in order to address the issue at its root. To re-sensitize these pathogens to antibiotics including methicillin, erythromycin, colistin, and carbapenem, CRISPR/Cas9 has targeted certain bacteria that contain antibiotic resistance genes like mecA, ermB, ramR, tetA, mqrB, and blaKPC. The most extensively researched genome-editing technology is S. pyogenes' CRISPR/Cas9, which consists of a Cas9 DNA endonuclease linked to tracrRNA and crRNA that may be systematically paired as sgRNA. Phage, plasmids, vesicles, and nanoparticles are the methods used to target CRISPR/Cas9 to bacterial cells. Recent developments on the molecular underpinnings of antibiotic resistance and the novel function of the CRISPR/Cas9 system in modifying these resistance mechanisms are discussed in this review. Additionally, methods for delivering this genome-editing technology to bacterial cells are examined. Furthermore, a few obstacles and potential opportunities are also discussed.

Key words: Antibiotics, bacteria, CRISPR/Cas9, and multidrug resistance

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Introduction

The prevalence of antibiotic-resistant illnesses is rising quickly since the 1928 discovery of antibiotics, which are now widely utilized as the preferred weapon against infections. Antibiotic-resistant strains of bacteria have emerged as a result of extensive antibiotic usage and abuse. Several variables, such as host biology, demographics, lifestyle, and the improper and frequent use of identical antibiotic combinations, contribute significantly to resistance sensitivity and the development of antimicrobial resistance (AMR) (Nath et al., 2022). It is crucial to discover some new medications to eradicate MDR complications. Other dangerous bacteria include drug-resistant *Shigella*, *Streptococcus pneumoniae*, *Campylobacter*, *Salmonella serotype typhi*, *M. tuberculosis*, *non-typhoidal Salmonella*, and extended-spectrum beta-lactamases. Other bacteria in this group include methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant Enterococci (VRE), multidrug-resistant *Pseudomonas aeruginosa*, and Enterobacteriaceae that produce ESBL. However, erythromycin-resistant group A streptococcus and clindamycin-resistant group B streptococcus are also among the strains of the dangerous bacteria (Kundar & Gokarn, 2022). Even though not all bacterial species are resistant to medicines, six main MDR bacteria—also referred to as ESKAPE bugs—have been found that mostly avoid the effects of antibiotics. The majority of nosocomial infections worldwide are caused by these bacteria, which include *Enterococcus fecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and Enterobacter species (Mulani et al., 2019). Various strategies have sometimes been

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used to address the issues caused by AMR. These strategies include phage treatment, antimicrobial peptides, vaccines, medication repurposing, anti-virulence chemicals, and more. Each of these approaches has some special qualities, but they also have drawbacks, which forces the researchers to look for some fresh, more effective options. Recently, the use of restriction endonucleases, RNA interference, and other genome-editing techniques has also been used to address MDR issues (Murugaiyan et al., 2022). Prokaryotes and archaea have well-known genome-editing defensive systems called CRISPR/Cas, which come in a variety of classes, kinds, and subtypes and protect against phages, plasmids, and mobile genetic elements (Schrader et al., 2020). Recent revisions to some of the molecular processes of AMR are examined in this article. The innovative methods of using CRISPR/Cas9 as a genome-editing tool to reduce the MDR are described in this context. Furthermore, methods for delivering this genome-editing tool to other bacteria are shown. Additionally, some of MDR's difficulties and opportunities are described.

The research's objectives are:

Consider the following crucial elements while planning your study on using CRISPR-Cas-based technologies to address antibiotic resistance in multidrug-resistant (MDR) bacteria:

i. Examine if CRISPR-Cas/Cas systems can be used to target and deactivate the genes responsible for MDR bacterial antibiotic resistance.

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ii. Consider how CRISPR-Cas may be used to create future antibiotics that specifically target and eliminate dangerous bacteria
iii. Conjugative plasmids, bacteriophages, and nanoparticles are among of the delivery systems that need be developed in order to improve the efficiency ofCRISPR-Cas in targeting MDR bacteria. There are
iv. CRISpen-based research techniques to prevent the emergence of antibiotic resistance by genetic mutation or adaptation.
v. Use in vitro and in vivo studies to evaluate the safety and efficacy ofCRISPR-Cas-based therapies for MDR infections to ensure that adverse effects are minimized.

Problem Statement:

Antibiotic resistance is a serious global health issue that raises the risk of morbidity and mortality and compromises the efficacy of existing treatments. Despite advances in antimicrobial research, the emergence of MDR bacteria has surpassed the development of new, powerful drugs. Conventional therapies are less specific and may sometimes have detrimental effects on the host microbiota. A potential alternative would be CRISPR-Cas-Cas technologies, which allow precision genome editing to disrupt resistance mechanisms and reestablish bacterial sensitivity to antibiotics. Effective delivery, probable CRISPR-Cas resistance, and ecological impacts on the whole population are still major challenges, nevertheless. These issues need to be addressed to fully recognize CRISPR-Cas as a ground-breaking therapy for antibiotic resistance.

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Significance of Study:

Particularly in multidrug-resistant (MDR) bacteria, the growing frequency of antibiotic resistance seriously tests global healthcare systems. Conventional antibiotics raise morbidity, death, and medical costs since they become less effective against bacteria with resistance mechanisms. This discovery is important since it offers a fresh creative genome editing method called CRISPR-Cas-based technology a means of fight against multidrug-resistant bacteria. Unlike traditional antibiotics, which typically act generally and indiscriminately, CRISPR-Cas systems directly target and destroy resistance genes inside bacterial genomes, so offering unequaled accuracy. This accuracy preserves the natural equilibrium of bacterial populations by means of less negative consequences on beneficial microorganisms. Furthermore, the programmable features of CRISPR-Cas offer an ecologically friendly and flexible antibacterial treatment by allowing a quick reaction to developing resistance mechanisms. Furthermore, the work addresses important field-related problems by means of conjugative plasmids, bacteriophages, and nanoparticles expanding the spreading modes of CRISPR-Cas-based technologies. These advances increase the security and accuracy of CRISPR-Cas applications. By enabling the development of scalable, tailored, and environmentally friendly drugs, this project has the potential to significantly impact public health and hence change approaches to handle antibiotic resistance.

Design of the Study

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This approach uses a whole experimental and theoretical framework to investigate the efficiency against multidrug-resistant bacteria using CRISPR-Cas technology. Identification of mobile genetic components and necessary resistance genes allowing multidrug-resistant bacterial strains marks the first phase in gene targeting and selection. One can find sequences fit for CRISPR-Cas targeting by means of genetic research and bioinformatics tools, so guaranteeing great specificity and efficiency. The main objective of the design is to improve methods of delivery for the integration of CRISPR-Cas structures into bacterial cells. The paper investigates more efficient distribution strategies including: Some bacteria might just pick up CRISPR-Cas tools from altered bacteriophages. Their encapsulation into nanoparticles provides their consistent and strong dispersion. Unique plasmids and horizontal gene transfer will enable CRISPR-Cas construct to spread over bacterial habitats. Under extensive in vitro testing, CRISPR-Cas systems are showing great success eradicating MDR bacteria, deleting resistance genes, and restoring antibiotic sensitivity. Further in vivo study in infection models supports complex biological systems to evaluate the safety, efficacy, and treatment possibilities of the advised treatments. Tests guarantee us that CRISPR-Cas technologies neither accidentally harm human cells nor non-target animals. Under observation is possible resistance to CRISPR-Cas-based therapy to forecast and lower problems with resistance development. Modern computational and statistical technologies help to confirm experimental results. Comparative study including contemporary antibiotics supporting the benefits of CRISPR-Cas technology.

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We aim to provide a strong and scalable platform for efficiently eradicating multidrug-resistant bacteria by tackling the technical, biological, and ecological aspects of CRISPR-Cas-based antimicrobial development.

Literature review

Allemailem (2024) contends that multidrug-resistant (MDR) bacteria significantly increase death rates and hinder the progress of medical technology, hence antibiotic resistance has become a major worldwide problem. Many ESKAPE infections have revealed alarming degrees of resistance to conventional treatments. Among the infections are species of Enterobacter, Klebsiella pneumoniae, Acinetobacter baumannii, Staphylococcus aureus, and Enterococcus faecium. First fit for scientific use in 2012, CRISPR-Cas/Cas9 is a genome-editing tool. This method helps one to target molecular level antibiotic resistance. If CRISPR-Cas-based treatments specifically target resistance genes; they might be able to minimize the inadvertent harm done to beneficial bacteria while preserving the efficacy of current antibiotics. Stressing the opportunities, challenges, and constraints of the most recent results in CRISPR-Cas-based therapeutics for multidrug-resistant bacteria, this work emphasizes their importance. Resistance against Antibiotics: Historical Context One might describe mechanisms of antibiotic resistance depending on the person as either natural or acquired. Some drugs have natural defense against them from inherent resistance found in bacterial DNA. Either this protective mechanism increases the efflux pump production eliminating medicines or lowers the drug molecule permeability. On the other hand, acquired resistance comes from either drug breakdown by enzymatic

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mechanisms, escape from the action of antibiotics, or alteration of antibiotic targets by means of horizontal gene transfer or spontaneous changes. These systems used together aggravates multidrug-resistant (MDR) challenges, which in turn makes the treatment plan execution more challenging and helps to distribute resistant strains in clinical and community settings. Unlike natural resistance encoded by bacterial chromosomes, acquired resistance can be produced by means of mutations or horizontal gene transfer mechanisms. Among the most crucial systems Darby *et al*. (2023) are target modification, the development of biofilm, inactivation of antibiotics, and superexpression of effluent pumps. More particularly, ESKAPE bacteria (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter species*) are the offenders behind nosocomial diseases for which there are limited therapeutic choices. Mulani et al. (2019) claim that some disorders hardly benefit from traditional medicines. Approaches Against MDR Effects Although phage therapy, antimicrobial peptides, and combinatorial pharmaceutical treatment are some of the conventional treatments used to attack multidrug-resistant bacteria, several of these approaches have particular drawbacks. Usually useless, these therapies have great expense of application, risk for toxicity, and fast rate of adaptability of bacteria to them. Apart from not being able to distinguish good from bad bacteria, they might disturb the microbial balance. Among the factors falling under this category are cost, negative consequences, and insufficient control of rapidly rising opposition. One area of likely contention is recent developments in genome-editing technologies such

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CRISPR-Cas/Cas9. Targeting and changing genes producing resistance, the CRISPR-Cas/Cas9 system restores bacterial susceptibility to medicines unlike more conventional methods. Applications for the Cas 9/CRISpen system Originally existing in the defensive mechanism of prokaryotes against phages and other genomic elements entering their genomes, CRISPR-Cas/Cas9 first Development early on revealed a bacterial immune system. 2012 saw Doudna and Charpentier restructure the system so that it could use single-guide RNA (sgRNA) for precision targeting and show that it was suitable for genome editing, essentially transforming the field of genetic research. Since single-guide RNA (sgRNA) leads the Cas9 nuclease to specific DNA locations, one can get customized cleavage. Among other things, Cas9/CRISpen takes use of MDR bacteria. Allemailem (2024) claims that a strategy aiming at genes such mecA (methicillin resistance) and ermB (erythromycin resistance) would assist to lower bacterial resistance. Reducing horizontal gene transfer between bacteria will depend on plasmids with resistance genes being eliminated. Re-sensitized to drugs such colistin and carbapenems (Dong et al., 2022), effective in order to enhance the efficacy of antibiotics are modified bacteria. Original methods for delivering Cas9/CRISpen directly connected with its efficacy is efficient distribution of CRISPR-Cas/Cas9 to bacterial cells. Strong cell walls provide bacterial cells remarkable resistance, unlike those of eukaryotic cells. Large molecules as CRISPR-Cas/Cas9 complexes present a tremendous difficulty from these cell membranes. Moreover, the absence of endocytosis channels makes internalization more difficult, so innovative methods of internalization including phage-mediated transfer or nanoparticle

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encapsulation are applied to meet the requirements for efficient targeting. The following are recent developments: Bacteriophages in precisely and efficiently allow CRISPR-Cas/Cas9 systems to be introduced into bacterial hosts. Studies by Wang et al 2021 show that lipid-based nanoparticles stop disintegration and aid to boost cell penetration. These nanoparticles feature CRISPR-Cas elements. Natural occurring bacterial vesicles will let CRISPR-Cas be transported and horizontally gene transferred (Rodolakis et al., 2023). Potential Future Prospectuses and Obstacles to be Overcoming Though CRISPR-Cas/Cas9 shows potential, against MDR bacteria it is not very helpful. Some research indicate that thick peptidoglycan coatings of gram-positive bacteria make it harder for CRISPR-Cas/Cas9 to be absorbed into their cell walls. Furthermore, present among some strains of bacteria are anti-CRISpen proteins, which reduce system efficacy by thus affecting its normal operation (Darby et al., 2023). Case studies may serve to expose impacts beyond the predicted results. Targeting *E. coli*, for example, unintentionally altered genes not resistant to the therapy, therefore distorting the outcomes of the treatment (Allemailem, 2024). Therapeutic usage of CRISPR-Cas will be able to grow should these problems be resolved. Among these include unanticipated results, issues with bacterial cell walls, and produced anti-CRISpen proteins. Bioinformatics is predicted to improve sgRNA design and aid to restrict unwanted alterations and limit Moreover, if hybrid delivery strategies combining phages and nanoparticles can overcome existing limitations, CRISPR-Cas may become useful from a medicinal standpoint. Case-based systems implemented in accordance with CRISPR-Cas-Based Technology. Originally found in bacteria as

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an adaptive immune system, CRISPR-Cas-Cas systems revolutionize genetic modification in precision and versatility. Two such genes, MecA linked to methicillin resistance and ermB, linked to erythromycin resistance, help combat MDR bacteria by targeting resistance genes! This is particularly crucial for recycling medications that have previously failed as CRISPR-Cas may switch off these genes and provide bacterial resistance back-off for present antibiotics. Eliminating plasmids allowing horizontal gene transfer is another smart way to handle the basic source of resistance reproduction. One may assist to disseminate CRISPR-Cas developments using present technology like as bacteriophage vectors, which are designed to especially target certain bacterial species. Giving CRISPR-Cas components straight into bacterial cells helps these phages lower off-target effects and thereby increase accuracy. lipid-based nanoparticles provide analogous protection of CRISPR-Cas complexes against breakdown and entry into bacterial populations resistant to chemicals. Allemailem, 2024; Given the challenges posed by bacterial cell walls, especially in Gram-positive strains with thick peptidoglycan coatings, these are very significant gains. Uses of RNA in Therapist CRISPR-Cas is another method for focusing on resistance genes; other RNA-based approaches consist of antisense RNA and RNA interference (RNAi). By means of their reduction of the transcriptional level of resistance genes, these medications may prevent the creation of proteins exhibiting antibiotic tolerance. Unlike genome editing, which permanently alters the DNA of the bacterium, RNA-based treatments could lower the environmental risks connected with genetic manipulation. Still, RNA-based treatments provide a significant barrier for us to

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pass. Two factors define their likely reach to bacterial cells: low absorption efficiency and sensitivity to breakdown in biological systems. Possible solutions for these issues might include coupling with peptides or nanoparticle carriers. Though they serve to reduce toxicity, every one of these techniques might increase intracellular absorption. When a little reduction of resistance genes is sufficient, RNA-based techniques show promise (Darby et al., 2023). This is true in spite of the challenges experienced. Tools That Share Often called "phage therapy," repurposing bacteriophages—usually fighting bacteria—to provide CRISPR-Cas or operate as stand-alone antibiotics Against multidrug-resistant bacteria, phages may more accurately reduce bacterial counts than traditional antibiotics. Main limitations still include low host specificity and phage resistance. Combining phage therapy with CRISPR-Cas with the benefits of both systems provides a symbiotic solution. Nanotechnology attracts a lot of interest as nanoparticles might carry and maintain antibacterial drugs or genetic tools like CRISPR-Cas to wipe out microorganisms. Their small size allows them to cross bacterial barriers; their surface may be changed to facilitate focused therapy. Recent research show that lipid-based nanoparticles might boost the efficacy of CRISPR-Cas delivery, therefore they provide a suitable approach to overcome resistance in clinical environments. Effective deployment of CRISPR-Cas technology into bacterial cells with tough cell walls still depends on delivery constraints. Future use of bioinformatics will assist to increase the targeting accuracy of single-guide RNA (sgRNA) designs, hence reducing off-target effects and increasing target precision. Currently under research is phage therapy, a hybrid delivery method for

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nanoparticles combined with limitations' cure. These CRISPR-Cas-based technologies can transform the treatment of diseases resistant to many different medications and boost the therapeutic use of antibiotics.

The Biology of Resistance Against Antibiotics

Antibacterial resistance, or MDR, is the capacity of bacteria to live in antibiotic surroundings, thought to kill them. Although the MDR is growing daily, concurrently rising two main categories into which the MDR falls. Genetic MDR results from the mutations in bacterial DNA or antibiotic resistance genes acquired from other bacteria. Clinically, this kind of MDR results in primary treatment failure, hence alternate antibiotic usage or new therapeutic techniques are necessary. Unlike genetic MDR, phenotypic MDR produces alterations in bacteria and diminishes within individual cells without any genetic modification. This kind of MDR against a particular antibiotic does not allow the full bacterial population to flourish generally (Wimmer& Beisel, 2020). In the event of phenotypic MDR in the presence of a particular antibiotic at or above minimum inhibitory concentration (MIC), the general population of bacteria is not allowed to develop. This type of AMR can manifest as a minimal killing rate for the bulk bacterial population, sometimes termed as “tolerance” (Hussain et al., 2019). Conversely, phenotypic antibiotic resistance usually known as "persistence" has a slower death rate than most of the bacterial population. Understanding the molecular underpinnings of MDR will enable one to find some creative ways to treat

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infectious illnesses. 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 (Liu C et al., 2017).

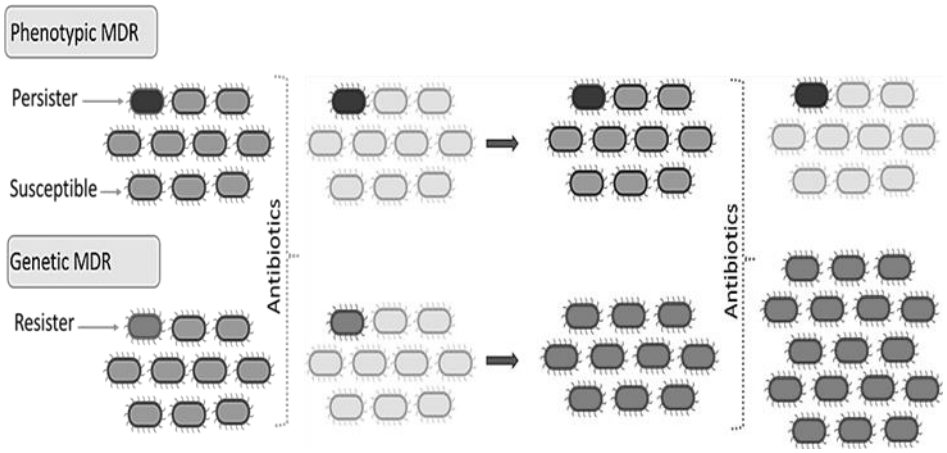

Figure 1: Shows the genetic and phenotypic MDR diagrammatically.

Although the phenotypic MDR bacterial cell has genetic characteristics with its siblings, its metabolic state allows it to withstand the first exposure to a particular antibiotic (dark grey cell). Nevertheless, this bacterium produces new offspring in such a way that the same percentage of bacteria are killed by a particular antibiotic on the second encounter. A mutant bacterium (red cell) with genetic MDR may be able to withstand the concentration of antibiotics to live, and these bacteria will continue to divide. Most of the sensitive bacteria (light grey cells) die when

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exposed to certain antibiotics. Even in the absence of a particular antibiotic, resistant bacteria continue to proliferate, and their offspring continue to have the caused mutation. Bacterial survival is unaffected by a second exposure to the same antibiotic and keeps growing (Shabbir MA et al., 2019). Understanding the biochemical activity of various antibiotics and the main mechanism by which bacteria evade the antibiotics' ability to kill them in various ways has advanced significantly. According to Rodrigues et al. (2019), the resistance mechanisms encompass a variety of actions, including the expression of resistance genes, downregulation or modification of porins to reduce the influx of antibiotics, antibiotic inactivation, overexpression of active efflux pumps, antibiotic target site modification, target bypass, and target protection. Recent developments in various technologies have uncovered the finer points of many resistance mechanisms. These include the function of intricate efflux systems that indicate likely pathways for the creation of inhibitors (Neil K et al., 2021).

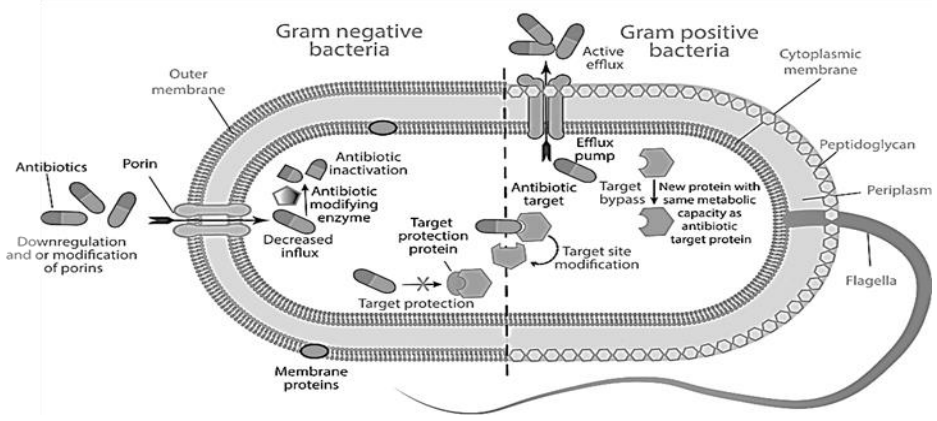

Figure 2: Overview of many molecular processes of MDR in bacteria.

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Reduced inflow of antibiotics results from downregulation of transmembrane proteins or structural changes in them. Mutation of genes encoding the protein target or modifications in the binding site causes the target site changes of certain proteins (Smith et al., 2019). The target bypass is the antibiotic attaching to a new protein without reducing its activity. Target protection proteins physically coupled with antibiotic target proteins releases it from antibiotic-mediated inhibition. Table 1 summarizes the mechanism of antibiotic resistance with examples of many proteins and enzymes along with their gene location. Various Strategies to Overcome MDR Combining combinatorial therapy, vaccines, drug repurposing, antibodies, antimicrobial peptides, phage therapy, anti-virulence compounds and drug-loaded nanoparticles (NPs) (Alav et al., 2021) will help to lower the MDR. Every one of these approaches has its drawbacks, which drives the search for fresh ideas with more effectiveness. Combining many drugs instead of one to achieve the synergistic impact to kill the germs by focusing on several locations is the basis of combinatorial treatment (Alav et al., 2021). The fast spread of MDR bacterial strains calls for combinatorial rather than monotherapy treatment instead of which is progressively insufficient to treat most bacterial infections (Alav et al., 2021). Monoclonal antibodies (mAbs) have also been used to address certain bacterial resistance (Al-Fadhli et al., 2024). Nevertheless, obstacles to their utilization include bacterial target selection (e.g., lipopolysaccharides have diverse serotypes) and breakdown by bacterial proteolytic enzymes (Al-Fadhli et al., 2024). RNA-based treatments include steric-blocking oligonucleotides and RNA interference (RNAi) to cure AMR species. These strategies target bacterial mRNA

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enzymatically by means of oligonucleotides, therefore eliminating genes causing the resistance phenotypes. Antisense RNA-based treatments have also given the capability to track genes in charge of MDR and growth promotion (Lee et al., 2020). But certain toxicity problems and poor intracellular absorption characterize RNA-based therapies (Sharma R et al., 2019). Using gene editing technologies such Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), which can precisely alter DNA of drug-resistant bacteria, the MDR problems have also been addressed (Li et al., 2020; Deng et al., 2019). TALENs and ZFNs are used against certain DNA sequences for their cleavage (Lee et al., 2020). Modern gene editing techniques now follow a fresh path thanks to TALENs and ZFNs. Further study is needed for optimization (Gonzalez AM et al., 2019; Patel A et al., 2021) despite significant constraints like complexity and off-target impacts.Aminoglycoside modifying enzyme AME; N-acetyl transferases AAC; 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.Delivery difficulties and effects have meant that these genome-editing techniques have not yet become generally popular. These techniques finally resulted in the creative use of genome-editing based on CRISpen/Cas for the fight against antibiotic resistance (Jones et al., 2020). Casa System and Crispen/Cas Methodologies Protecting archaea and bacteria against phages, plasmids, and other mobile genetic elements (Zhang et al., 2019), the CRISpen/Cas system is a novel

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genome-editing technology acting as an adaptive immune system. About 75% of archaea and 36% of bacteria have the CRISpen/Cas system in their genome (Doudna & Charpentier, 2014). Recent research shows an inverse relationship between antibiotic resistance—including ESBL and carbapenemase synthesis in antibiotic-resistant bacteria like Klebsiella pneumoniae—and the existence of the CRISpen/Cas system (Lee et al., 2020). Furthermore, observed in Enterococcus faecalis and Enterococcus faecium are the frequency and probable link of CRISpen/Cas with antibiotic resistance. Comprising short flanking sequences known as spacers and repetitive sequences, the CRISpen/Cas genetic loci integrate the CRISpen array. In CRISpen, protospacers—derived from invading plasmids or phage DNA—form the spacers. Key players in the CRISpen system, Cas proteins are encoded upstream of the CRISpen array (Jinek et al., 2014). Either single or several loci on a chromosome might allow the CRISpen array to be grouped (Wiedenheft et al., 2012). When Doudna and Charpentier effectively showed in 2012 the utilization of the CRISpen/Cas9 system with a chimeric single guide RNA (sgRNA), a major progress was reached. Joining CRISpen RNA (crRNA) and transactivating CRISpen RNA (tracrRNA) simultaneously results in the sgRNA (Doudna & Charpentier, 2014). There are two primary classes, six kinds (I to VI), and 33 subtypes to the CRISpen/Cas systems. Differences in Cas protein composition define this categorization, which is compiled in Figure 3 (Makarova et al., 2015; Barrangou & Marraffini, 2014).

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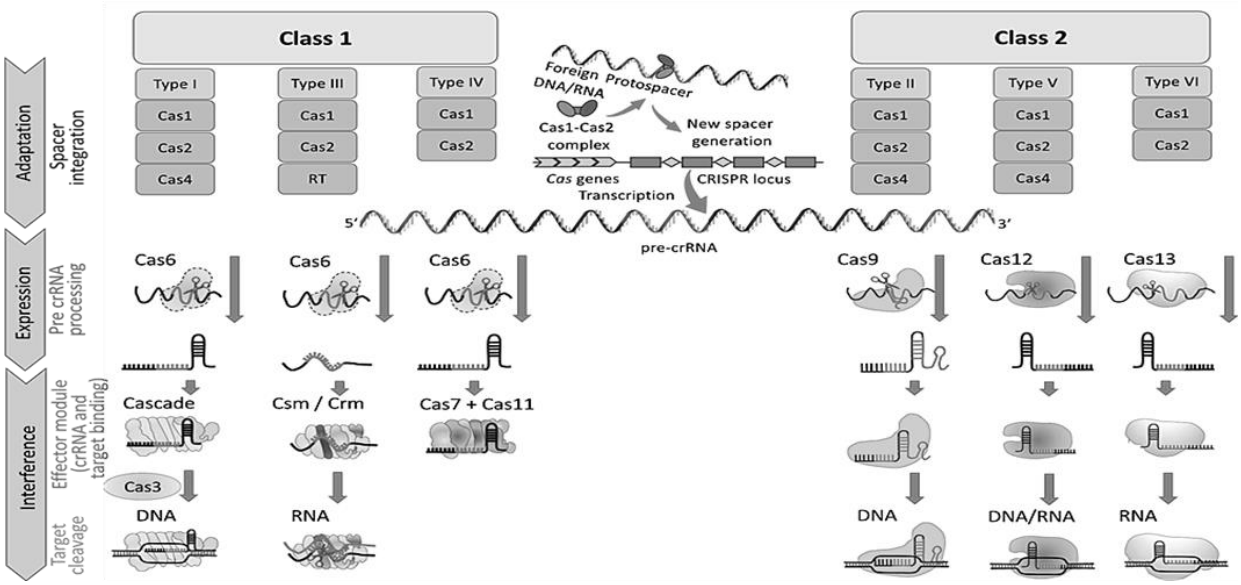

Figure 3: Bacterial CRISpen/Cas system categorization and applications. Functional components of the CRISpen/Cas system are illustrated and Cas effectors are categorized based on the general organization.

With sixteen subtypes and combining effector modules made of several Cas proteins, class 1 in this scheme of CRISpen/Cas categorization consists of types I, III, and IV. These effector modules help pre-crRNA (Makarova et al., 2015) to be processed and acted upon as well as for crRNA determination. Class 2 encompasses types II, V, and VI, which have seventeen subtypes each and each type consists of a single, big, multi-domain crRNA-binding protein. This class deals with pre-crRNA processing in certain variations and interference in all variants (Barrangou & Marraffini, 2014; Jinek et al., 2014). Every kind of the CRISpen/Cas system has a different protein composition for the expression and interference stages (Doudna & Charpentier, 2014). The essential purpose of this

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genome-editing tool stays the same even if the CRISpen/Cas system is rather diversified. Three basic phases comprise it: adaptation, expression, and interference (Figures 3 and 4). Exogenous DNA pieces are included into the bacterial CRISpen array during the adaption phase; subsequently, the expression phase maturates CRISpen-RNA (crRNA) from the acquired spacers (Wiedenheft et al., 2012; Doudna & Charpentier, 2014). The interference phase is the recognition and binding of crRNA to complementary nucleotide sequences in the attacking genetic elements, which causes Cas nuclease (Mojica et al., 2009; Zhang et al., 2019) cleaving. [Figure 4]. Because of its adaptability, simplicity, specificity, and efficiency—among the many type II CRISpen/Cas9 systems—the S. pyogenes-derived CRISpen/Cas9 system has been much investigated (Jinek et al., 2014; Cong et al., 2013). CRISpen/Cas9 System Structural Analysis Since the interference step of the CRISpen/Cas9 produced from S. pyogenes (Spy) only needs the Cas9 protein, this version of the CRISpen/Cas9 is the most investigated one (Jinek et al., 2014; Doudna & Charpentier, 2014). Comprising Cas9 protein and single guide RNA (sgRNA, this system is a ribonucleoprotein complex). With 1368 amino acid residues, the 160 kDa DNA endonuclease enzyme Cas9 can cut both strands of double-stranded DNA (Doudna & Charpentier, 2014; Cong et al., 2013) .While RNaseIII (Jinek et al., 2014; Zhang et al., 2019) carries out maturation of pre-crRNA to sgRNA, this genome-editing tool also needs Csn2, Cas1, and Cas2 for the DNA acquisition step.Two lobes make up Cas9 endonuclease: the recognition (REC) lobe (residues 56–718) (Doudna & Charpentier, 2014; Zhang et al., 2019) and the nuclease (NUC) lobe, which

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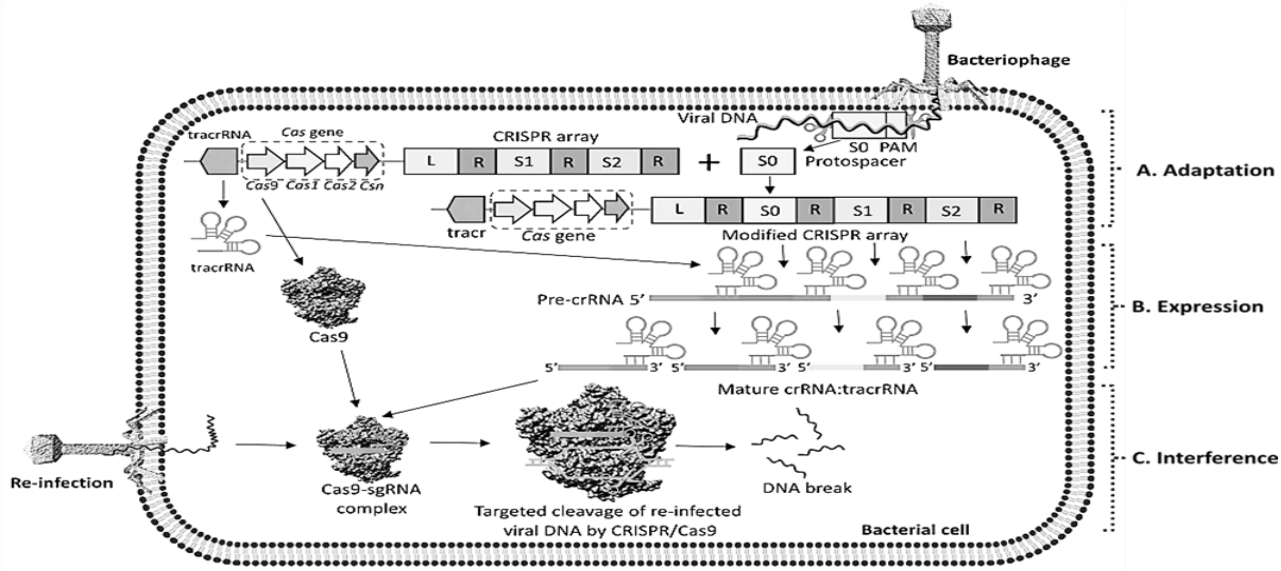

Figure 4: Bacterial cell CRISpen/Cas9 system mechanism of operation

The three phases consist in: (A) modification for including foreign DNA into CRISpen loci. During expression, CRISpen loci are transcribed, and the Cas9/RNase III complex further processes the resultant pre-crRNA to produce mature crRNA that links with tracrRNA to make sgRNA. The CRISpen/Cas9 complex cleaves new phage-infected DNA with any sequence matching the CRISpen spacer of sgRNA during the interference stage (Jinek et al., 2014; Doudna & Charpentier, 2014). Two endonuclease domains—the RuvC domain (residues 1–55, 719–765, and 910–1099) and the HNH domain (residues 766–909) define Cas9. Whereas the RuvC domain cleaves the non-target strand (Jinek et al., 2014; Zhang et al., 2019), the HNH domain oversees cutting the target DNA strand. Near the N and C termini of the HNH domain, two important hinge sites—linker L1 and L2—help to enable crosstalk between the HNH and RuvC domains

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(Doudna & Charpentier, 2014; Weng et al., 2019). Crucially for starting Cas9 binding to target DNA (Zhang et al., 2019), the PAM-interacting (PI) domain spans residues 1100 to 1368 and imparts PAM specificity. Positively charged residues between the NUC and REC lobes stabilize the negatively charged sgRNA:DNA hybrid upon DNA binding (Makarova et al., 2015; Jinek et al., 2014). The positively charged amino acid residues in the linker sites (L1 and L2) between the HNH and RuvC domains stabilize the non-target DNA (Weng et al., 2019). At various phases of its activity, conformational changes validated by X-ray crystallography studies constitute the action mechanism of CRISpen/Cas9. The structures include free Cas9 (PDB 4CMQ), sgRNA-bound Cas9 (PDB 4ZT0), Cas9 in complex with target DNA and incomplete non-target DNA with PAM (PDB 4Un3), and Cas9 in association with both target and complete non-target DNA (PDB 5F9R). Resolutions of 3.09 Å and 2.9 Å respectively were achieved for the apo Cas9 and sgRNA-bound Cas9 structures (Doudna & Charpentier, 2014). The REC domain experiences a notable rearranging upon sgRNA binding, including a 65 Å shift in the REC III domain to fit sgRNA (Zhang et al., 2019; Jinek et al., 2014). Following interaction of the target DNA and PAM with the incomplete non-target strand (Weng et al., 2019), the REC II domain undergoes yet further change. The Cas9: sgRNA complex following PAM recognition helps foreign DNA to melt and generates DNA: RNA hybrids (Zhang et al., 2019; Weng et al., 2019). CRISpen/Cas9's mechanical action Three steps define the mechanical activity of the CRISpen/Cas9 system: adaptation, expression, and interference (Jinek et al., 2014; Doudna & Charpentier, 2014; Weng et al., 2019). About 30 bp

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of invading foreign DNA is incorporated into the CRISpen locus leader side during the adaption step (Makarova et al., 2015; Zhang et al., 2019).

Three-dimensional structure of SpCas9-sgRNA-DNA ternary complex figure 5. Taken from the Protein Data Bank (http://www.rcsb.org, PDB ID: 4OO8), corrected by UCSF Chimera, (A) ribbon depiction of the Cas9-sgRNA-DNA complex at two distinct orientations. (B) Domain organization reflecting allocated amino acid residue counts for many protein domains. The step of adaptation consists in the inclusion of a protospacer adjacent motif (PAM) sequence from the host genome. Transcript of spacers from the CRISpen locus happens during the expression step. Combined with Cas9, sgRNA—a mix of tracrRNA and crRNA—has greatly increased genome editing (Jinek et al., 2014; Zhang et al., 2019). Furthermore, the PAM sequence is very important as it helps Cas9 to identify the target DNA sequence, thus guaranteeing the accuracy of the system (Doudna & Charpentier, 2014; Zhang et al., 2019). Studies on self-targeting spacers in CRISpen/Cas systems have shown that if the DNA repair mechanism is delayed, bacterial death results (Makarova et al., 2015). This suggests that reprogramming Cas nucleases might aid in targeting certain bacterial populations, therefore offering a means to either selectively kill or re-sensitize bacteria for antibiotic treatment (Doudna & Charpentier, 2014). Usually three base pairs upstream of the PAM region, Cas9 operates to produce a double-stranded blunt cut when sgRNA has enough complementarity with the target DNA. With blunt ends (Jinek et al., 2014; Zhang et al., 2019), Cas9 possesses two nuclease domains: the HNH domain cleaves the complementary DNA strand and the RuvC domain cleaves the non-

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complementary strand generating a double-stranded break (DSB). Two main processes underpin DSB repair: homology-directed repair (HDR) and non-homologous end joining (NHEJ). Operating without a homologous DNA template, NHEJ is very effective for gene disturbance by deletions or insertions. Less efficient HDR depends on a donor DNA template and is appropriate for exact gene changes (Doudna & Charpentier, 2014; Makarova et al., 2015). Targeting certain genes associated to virulence, multidrug resistance (MDR), or other important pathogenic features, the sgRNA may be customized. This lets the CRISpen/Cas9 system cause death or suppression of development in target organisms. Moreover, it may be designed to suppress target gene transcription and eradicate plasmids causing antibiotic resistance (Doudna & Charpentier, 2014; Zhang et al., 2019). Novel Strategies to Address MDR Based on Cas9/CRISpen Genome Editing Now generally seen as a viable next-generation technique for treating infectious illnesses, especially those driven by antibiotic-resistant (AMR) microorganisms, CRISpen/Cas9-based genome editing is Re-sensitizing pathogens to medicines and managing the proliferation of MDR bacteria (Makarova et al., 2015) depends on the position of the targeted gene. Double-stranded breaks of target DNA caused by CRISpen/Cas9 figure 6 Either NHEJ or HDR will allow one to fix the breaks. While HDR allows exact nucleotide replacements when a donor DNA template is available, allowing for particular gene changes, NHEJ-mediated repair causes gene disruption by either deletion or insertion of DNA sequences. Two basic techniques may be employed with Crispen/Cas9: gene-oriented and pathogen-targeted ones. Under the pathogen-targeted strategy, the system is guided to particular areas of

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the bacterial chromosome, therefore eradicating particular pathogenic strains and destroying bacterial cells. Conversely, the gene-based strategy targets antibiotic resistance genes found on plasmids, therefore rendering drugs once again sensitiveible to bacteria. Although less well-defined, the gene-focused strategy may help to generally lower antibiotic resistance in microbial populations whereas the pathogen-oriented approach is useful on specified infections and strains of mixed microbial pathogens. Unlike traditional antibiotics, the CRISpen/Cas9 system can differentiate between harmful and symbiotic bacterial strains by means of its exact targeting mechanism. Studies have shown, for example, the transformation of *Escherichia coli* and *Staphylococcus aureus* using a plasmid that codes Cas9-driven RNA, therefore targeting and destroying antibiotic resistance genes (Liu et al., 2017). Clinical studies have produced encouraging findings albeit remaining in early stages. In certain S. aureus clinical isolates, for instance, therapy with modified crRNA and Cas9 targeted the methicillin resistance gene (mecA), hence reducing the illness severity (Zhang et al., 2019). Targeting the ermB gene—which causes erythromycin resistance—in E. faecalis similarly helped to lower intestinal colonization of resistant strains. Klebsiella pneumoniae, an ESKAPE pathogen well-known for their multidrug resistance (MDR), was also investigated using the genome-editing method. Targeting the genes mgrB, tetA, and ramR affected the susceptibility of carbapenem-resistant K. pneumoniae to colistin and tigecycline (Wang et al., 2021). Another approach used was pheromone-responsive plasmid (PRP) delivering CRISpen/Cas9 to MDR Enterococcus faecalis. Targeting antibiotic resistance genes like tetM (tetracycline resistance) and ermB

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(erythromycin resistance) this approach helped to lower antibiotic resistance both in vitro and in vivo (Chen et al., 2021). Targeted genome editing caused changes in the lacL gene, therefore compromising antibiotic resistance in a strain called E745 and implying possible usage in other Gram-positive bacteria (Smit et al., 2019). One fresh approach was to target MDR *E. coli* in mice models by employing CRISpen/Cas9 on a conjugative plasmid (TP 114), therefore obtaining over 99.9% clearance in the gut flora. This approach cleared Citrobacter rodentium infections with equally success. Researchers employed CRISpen/dCas9 to downregulate genes involved in wall teichoic acid production in S. aureus, thus increasing S. aureus's susceptibility to lysostaphin, so overcoming bacterial resistance to antimicrobial drugs like lysostaphin (Li et al., 2022). Furthermore, tailored to precisely cleave carbapenemase genes (blaKPC, blaNDM, blaOXA-48) in carbapenem-resistant Enterobacteriaceae (CRE), was a CRISpen/Cas9-based plasmid-curing system (pCasCure). This method increased sensitivity to carbapenem antibiotics (Zhang et al., 2019) showing more than 94% effectiveness in lowering the carbapenemase gene presence. Novel Targeting Strategies for Cas9 Delivery in CRISpen Delivering CRISpen/Cas9 to bacterial cells presents difficulties particularly as the complex must to pass the bacterial cell wall. Phage, plasmid, nanoparticle, and extracellular vesicle-based techniques have been investigated among other delivery approaches by researchers. Natural predators of bacteria, phages may effectively bring their genetic material into bacterial cells. Developed to provide the genome-editing technology are modified phages including CRISpen/Cas9. Attaching to bacterial receptors, including pili or

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lipopolysaccharides, phages transfer the CRISpen constructions, therefore altering the target genes to lower antibiotic resistance. These focused strategies provide fresh directions for bacterial infection management and reflect the cutting edge in overcoming antibiotic resistance, hence lowering the load of MDR infections.Using a plasmid containing the CRISpen/Cas9 genome-editing technology aiming at MDR genes, *E. coli* was transformed. This approach produced an approximately one-thousand-fold decline in transformation efficiency when selective agents were present. These results set the stage for further studies using phages to bundle CRISpen/Cas9 vectors targeted targeting antibiotic resistance genes, thereby rapidly eradicating certain bacteria. Likewise, phage-encoded CRISpen/Cas9 targeted a virulent strain of *Staphylococcus aureus* showing drug resistance. This method shown species-specific targeting and death of bacterial. An in vivo mouse skin colonization infection model was created in order to bolster this creative solution even further. In this scenario, the topical administration of phages assisted by CRISpen/Cas9 greatly slowed down the bacterial colonization expansion. These results taken together show that although non-targeted bacteria remain unharmed, selectively targeted pathogenic bacteria may be destroyed using CRISpen/Cas9 antibacterial tools. Developing antibacterial treatments that maintain intestinal microbiota and other surroundings depends especially on this method.

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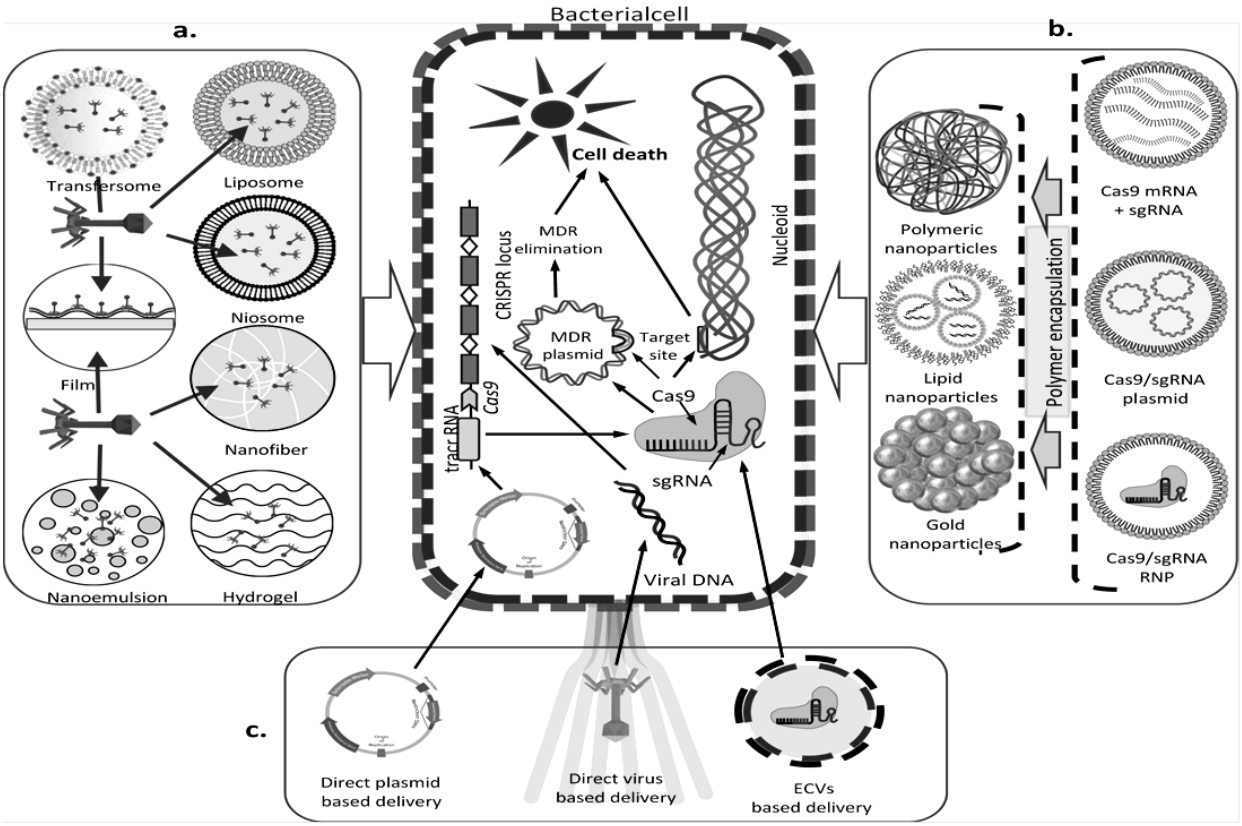

Figure 5: Several approaches for delivering CRISpen/Cas9 genome-editing technology in MDR bacteria. a. Techniques of bacteriophage encapsulation to produce various forms of Nano formulation for aiming at certain bacterial kinds. b. Different approaches to target the MDR bacteria using CRISpen/Cas9 components delivered as mRNA, plasmids, or RNPs or encapsulated polymeric NPs, lipid NPs, or gold NPs. c. CRISpen/Cas9 system is likewise directly targeted as plasmid-based delivery, viral-based delivery, or extracellular vesicle-based delivery to MDR bacteria to execute cell killing activities. Alginate hydrogel has been used in a parallel approach to provide the CRISpen/Cas9-phage complex, therefore

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lowering soft tissue infection with increased anti-biofilm effects over time. The CRISpen/Cas9 system may demonstrate great effectiveness against MDR activity with appropriate Nano formulation and bacteriophage encapsulation techniques; nonetheless, practical adoption of this new antimicrobial resistance treatment remains difficult. Conjugative Plasmids Oversaw Delivery

Apart from phage-mediated delivery strategies, other delivery techniques may be used to aim at the CRISpen/Cas9 system in MDR bacterial cells. Using conjugative plasmids as delivery vehicles lets genetic material be transferred across bacterial cells. Enterococcal antibiotic resistance has been much lowered using the CRISpen/Cas system supplied by plasmids. Carbapenem-resistant Enterobacteriaceae has also been used the plasmid-based technique to remove blaKPC and blaNDM and re-sensitize these bacteria to carbapenems with favorable results. By spreading the system to additional bacteria, plasmid recipient bacteria help to increase the use of this genome-editing technique to fight genes resistant to drugs. Targeted plasmid-based CRISpen/Cas delivery has been successfully used to Gram-negative Enterobacteriaceae and *E. coli* to stop drug resistance from proliferating. Re-sensitising pathogens to antibiotics and providing immunity against mcr-1, Dong et al 2022 developed conjugative CRISpen/Cas9 targeting the colistin resistance gene (mcr-1) in *E. coli*. With different MCR-1 plasmids, the recombinant plasmid pMBLcas9-sgRNA maintained its capacity to transfer into *E. coli*, thereby effectively removing MDR plasmids. Plasmid conjugation lacks receptors unlike phage-based delivery, which depends on certain receptors on bacterial cells for attachment. This makes plasmid-based distribution

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better because changes in bacterial receptors that can provide resistance to phage-mediated delivery have emerged. Among the advantages include biocompatibility, reduced immunogenicity, surface functionalization, and greater safety than using viral vectors. CRISpen/Cas delivery has made use of inorganic and cationic polymer-based nanoparticles. For example, effectively injected into MRSA a cationic polymer-based CRISpen combination comprising Cas9 and crRNA targets the methicillin-resistant gene, therefore acting to kill bacteria. Though problems concerning optimizing encapsulation rates and effective delivery to certain pathogens like Mycobacterium TB, which has a highly impenetrable cell wall, the delivery of CRISpen/Cas components utilizing nanoparticles is still in its early years.

Future Prospects and Challenges for Minimizing MDR with CRISpen/Cas9 Though CRISpen/ Cas9 genome-editing technology has great promise to fight multidrug-resistant (MDR) bacteria, its use in this sector is beset with difficulties. Many human diseases, including Burkholderia species, Mycobacterium TB, and Salmonella enterica, are intracellular pathogens living in host cells, so they are more difficult to remove using genome editing led by CRISpen/Cas9. Other approaches are thus required to improve the delivery of CRISpen/Cas9 systems to these intracellular infections. Although these techniques were created for eukaryotic cells, it is crucial to find if liposomes and phages can efficiently transfer conjugative plasmids, phage complexes, and nanoparticles (NPs). While CRISpen/Cas9 has shown successful in eradicating bacterial cells, certain colonies have been seen to persist by avoiding genome targeting. Among the elements

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generating resistance to this genome-editing tool are anti-CRISpen (Acr) gene of the host genome and spontaneous mutations in the spacer, Cas, or target regions. If such resistance is to be avoided, further research has to concentrate on stopping these Cas gene or crRNA spontaneous mutations. Target sequence changes generating variations of genes resistant to antibiotics significantly challenge the efficacy of CRISpen/Cas9.Using CRISpen/Cas9 for effective genome editing has some disadvantages, chief among them off-target effects—especially in vivo. Because of its size, Cas9—derived from Streptococcus pyogenes—SpCas9—is challenging to package into adeno-associated virus (AAV) vectors. An interesting approach to handle MDR is combining bioinformatics with CRISpen/Cas9. Design of sgRNAs and bacterial genome decoding call for bioinformatics. Innovative computer tools and sophisticated algorithms let researchers examine the evolution of resistance, evaluate antibiotic resistance, and create novel drugs by analyzing enormous volumes of data. Possibly more beneficial than traditional antibiotics are Crispen/Cas genome-editing techniques. Given more than 33 subtypes identified, it is thought that future studies might use more efficient CRISpen/Cas kinds to more successfully fight MDR. Additionally, expected to be developed are new and creative CRISpen/Cas9 delivery techniques that will allow more exact genome editing in many bacterial strains and solve MDR issues.

Conclusion

The misuse of antibiotics in healthcare has seriously affected human health as well as helped MDR bacteria to evolve and spread over the world. A potential approach

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to fight MDR without sacrificing helpful bacteria is CRISpen/Cas9 therapy of antibiotic-resistant bacteria. Targeting any MDR gene is achievable because to CRISpen/Cas9's great specificity, adaptability, efficiency, and programmability, hence perhaps addressing antibiotic resistance issues. Still in its infancy, this bacterial genome-editing technique suffers with effective targeting inside bacterial cells. Targeting specificity for CRISpen/Cas9 benefits from continuous study on phages, plasmids, and nanoformulations. Effective sgRNA, improving the decoding of bacterial targets, and creating unique Cas9 variations to lower off-target effects all depend on the usage of well-crafted bioinformatics tools as well. Significant prospective therapeutic uses for the CRISpen/Cas9 technology to solve MDR-related issues.

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