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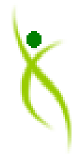

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Review on Anti-Biofilm Potential of CRISPR-Cas System on Various Pathogens

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Abstract: The development of bacterial biofilms entails regulatory as well as signalling mechanisms that regulate the shift from a mobile to a stationary state of existence, the production of the extracellular polymeric matrix, and the progression of 3-dimensional biofilm formation with emphasis on how easily they may be created and how crucial they are in biological, ecological, and industrial settings, biofilms are the subject of extensive research. Biofilms and a variety of pathogenic human disorders are frequently linked. Since bacteria in biofilms can resist antibiotics, the immune system, and other treatments, biofilm infections are typically long-lasting. Many prokaryotes include CRISPR-associated proteins (Cas), a solid adaptable immunological system that may be programmed to damage the bacterial genomes and induce cell death. Short palindromic repeats that are grouped and adequately spaced together make up CRISPR-Cas. In light of this, CRISPR-Cas can be seen as an exciting strategy to address and overcome antibiotic resistance. Furthermore, the CRISPR-Cas system can create "precise antimicrobials" that target bacterial infections according to specific DNA sequences. This CRISPR-Cas technique is susceptible to drug-resistant microorganisms due to its selective targeting of the genes involved in biofilm formation, pathogenicity, and antibiotic resistance. However, this method requires potent vectors for the CRISPR-Cas system to access the bacterial genomes. As vectors, genetically engineered Phage, liposomes, and lipid-mediated nanoparticles are exciting options. This technique has been used to prevent extracellular and intracellular from forming biofilms. The most current developments in creating innovations and possible advantages of the various CRISPR-Cas delivery methods for the deliberate eradication of bacterial pathogens will be covered in this review, focussing mainly on the anti-biofilm potential, which is found to be one of the primary causes of the difficulty of irradiation of Multi-drug resistant bacteria. Additionally, each distribution system's positive aspects are highlighted, along with its challenges and potential for advancement in the future.

Keywords: biofilm, biofilm-residing bacteria, CRISPR-Cas system, virulence, phages, liposomes, conjugative systems.

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1. INTRODUCTION

Biofilms, the most prevalent stage of bacterial life in nature, play a significant role in the prokaryotic life cycle. Almost every abiotic or biotic surface can support its formation[1-3]. Biofilms are made up of a syntrophic consortium of microorganisms that are embedded in a self-made matrix of extracellular biopolymers. These biofilms offer protection, interact with the environment, encourage quorum sensing among bacterial cells, and improve the ability of microorganisms to disperse from microbial clusters and colonize new niches[4,5]. The pathogenic bacteria that produce biofilms include *Klebsiella pneumonia*, *Enterococcus faecalis*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, and other gram-negative bacteria[6]. They are frequently associated with chronic infections, thought to be harmful to human health, and typically resistant to antibiotic treatments[7,8]. However, non-pathogenic biofilm-forming bacteria such as *Pseudomonas extremorientalis*, *Paenibacillus peoriae*, and *Streptomyces cirrus* can successfully defend plants against disease[9,10], increase plant development, and encourage symbiotic relationships between mycorrhizal fungi and plant roots[11].

1.1. Composition of microbial biofilms

A biofilm is a structured collection of bacteria grows inside an extracellular polymeric matrix and affixed to a live surface in an irreversible manner[12,13]. Biofilms cannot be removed unless they are rapidly rinsed. Extracellular polymeric compounds are created during the biofilm's adhesion phase to the surface. The biofilm is typically between 0.2 and 1.0 mm thick. However, it is no larger than 10 to 30 nm in size[14]. The extracellular matrix, which makes up the majority of the biofilm's volume and is typically composed of proteins (>2 per cent), polysaccharides (1-2 per cent), DNA and RNA molecules, ions (bound and free), and 97 per cent water, makes up the remaining volume. Microorganisms typically comprise 5-35 per cent of the biofilm's volume. The water content of a biofilm is attributed to the flow of essential nutrients inside it[15,16]. Further, the extracellular matrix is a scavenging mechanism to remove crucial nutrients and minerals from the environment and trap them.

1.2. Steps of biofilm formation

The first step in forming a biofilm is adhesion to a surface, irrespective of being living or not, this attachment will produce a microcolony, which will give rise to three-dimensional structures, and it will end with separation after maturation. Next, quorum sensing, unique signalling between the microorganism's cells, is required. The transcription of specific gene sets necessary for biofilm production differs from planktonic forms of the same microbial species. Finally, the extracellular polymeric material matrix's visco-elastic characteristics are responsible for the mechanical stability of biofilm[17].

1. The first contact and attachment to the surface have been established via appendages like pili and flagella, which is the first step in creating a biofilm. Other physical forces, like the Vander Waals and electrostatic interaction forces, can also hold them together. These appendages strengthen the connection between the attachment surface and the bacteria. Additionally, during the creation of a biofilm, microbial cells connect to a surface (adhesion) and interact with one another (cohesion)[18,19]. Since it reduces the repulsive force between the bacterium and the character, surface hydrophobicity can also contribute to better microbe attachment[20].
2. Micro-colony formation is the second stage of biofilm formation. A phase of microbial cell multiplication and division starts once microorganisms connect to the surface. This process usually begins inside the extracellular polymeric substances by specific chemical signalling. The establishment of a syntrophic association an affiliation of two or more metabolically distinct bacteria for using particular substrates as a source of energy, dependent upon one another can be fully facilitated by biofilm[21].
3. Maturation and architecture comprise the third step in the creation of a biofilm. Here, auto-inducer signals are used by microbial cells to connect with one another[22,23]. In addition, quorum sensing is made easier by this autoinducers[24]. At this stage of development, specific gene products thought to be necessary for synthesizing extracellular polymeric compounds are expressed.
4. Dispersion or detachment of the biofilm is the fourth step in creating a biofilm. First, the biofilm's microbial inhabitants rapidly replicate and disperse to transit to a motile state. Next, a natural pattern of dissociation takes place[25]. Finally, various saccharolytic enzymes, including hyaluronidase produced by *Streptococcus equi*[26], are produced by microbial communities within the biofilm during the detachment process that transfers the bacteria into a new colonization zone. Infections spread more readily when microbial cells separate and move to a new place[27].

1.3. Pathogenesis of disease: The role of biofilm

A decreased metabolic rate along with a reduced rate of cell division may arise from the bacteria living inside biofilms, altering their gene expression, metabolism, and protein synthesis in response to ambient anoxia and nutrients[28-30]. These alterations make the bacteria more resilient to antimicrobial treatment in addition to inactivating the anti-bacterial targets or lowering the requirements. Additionally, by developing biofilms, the bacteria can subvert the host's immune system[31]. A biofilm infection can simultaneously activate the host's innate and acquired immune systems. Still, neither of these systems can eradicate the biofilm pathogen and speed up collateral tissue damage[32]. Therefore, biofilm-related disorders are typically recurrent infections that progress slowly, are seldom treated by the immune system, and have variable responses to antimicrobials. Biofilms can result in severe chronic disorders and are associated with more significant morbidity and mortality rates. Therefore, a better technique is required to prevent pathogenic organisms from forming biofilms. Treating biofilm infections is particularly challenging when dealing with antibiotic resistance and tolerance. Antimicrobial agent deactivation in the outer layers of the biofilm by binding to matrix components or enzymatic modification restricted antimicrobial agent diffusion in the biofilm matrix, and the presence of niches in the biofilm with less responsive cells, such as starved cells and persisted cells, have all been proposed as mechanisms for antimicrobial resistance and tolerance[33-5]. We urgently require novel approaches that can prevent these resistance mechanisms. The CRISPR-Cas system is one option that is now generating much interest. Thus, the review focused on the clinical significance of biofilm

formation and increased virulence; further CRISPR-CAS system as an alternative approach for biofilm inhibition has been highlighted with particular emphasis on CRISPR-Cas Delivery methods.

2. CRISPR-CAS SYSTEM TO INHIBIT BIOFILM FORMATION

Numerous bacterial as well as archaeal genomes contain "Clustered and Regularly Interspersed Short Palindromic Repeats (CRISPR)" and CRISPR-associated (Cas) genes. Variable sequences found in prokaryotes have been employed as an additional typing technique in clinical, epidemiological, and evolutionary investigations since the usual CRISPR loci were identified in the 1980s, long before their physiological relevance was understood. Ultimately, the explanation of CRISPR-Cas as an adaptive immune system was made possible by the realization that CRISPR spacers match sequence pieces of mobile genetic components. Small CRISPR RNAs serve as the primary building blocks of this particular prokaryotic defence system, directing nucleases to target the complementary nucleic acids of invasive viruses and plasmids. CRISPR-Cas systems can affect bacterial pathogenicity and other genetic changes through two non-exclusive pathways[36]. On the one hand, CRISPR-Cas protection can lessen the possibility of bacterial pathogenicity since mobile elements can transfer foreign DNA containing possible virulence factors such as toxins or antibiotic-resistance genes. On the other hand, however, gene expression is controlled, which may cause the expression underlying virulence genes to decrease. This effectively stops many bacteria from building biofilms (figure 2). Three steps make up the CRISPR-Cas immunity system (figure 1):

1. Spacer acquisition or adaptation, the initial step [37], adds the sequences of recognized spacers to the CRISPR array.
2. In the second phase, referred to as biogenesis or CRISPR RNA (crRNA) expression, RNA polymerase transcribes pre-CRISPR RNA (pre-crRNA) (RNAP). Then, distinct endoribonucleases separate these pre-crRNAs into imperceptible crRNAs. Based on the role of crRNA, these RNAs are also referred to as guide RNAs[38,39].
3. Interference, the third and last step, is where interference happens[40]. CrRNAs recognize and shape base pairs exclusive to international RNA/DNA that are almost perfectly complementary. This makes it easier to separate the complex of the foreign nucleic acid and crRNA.

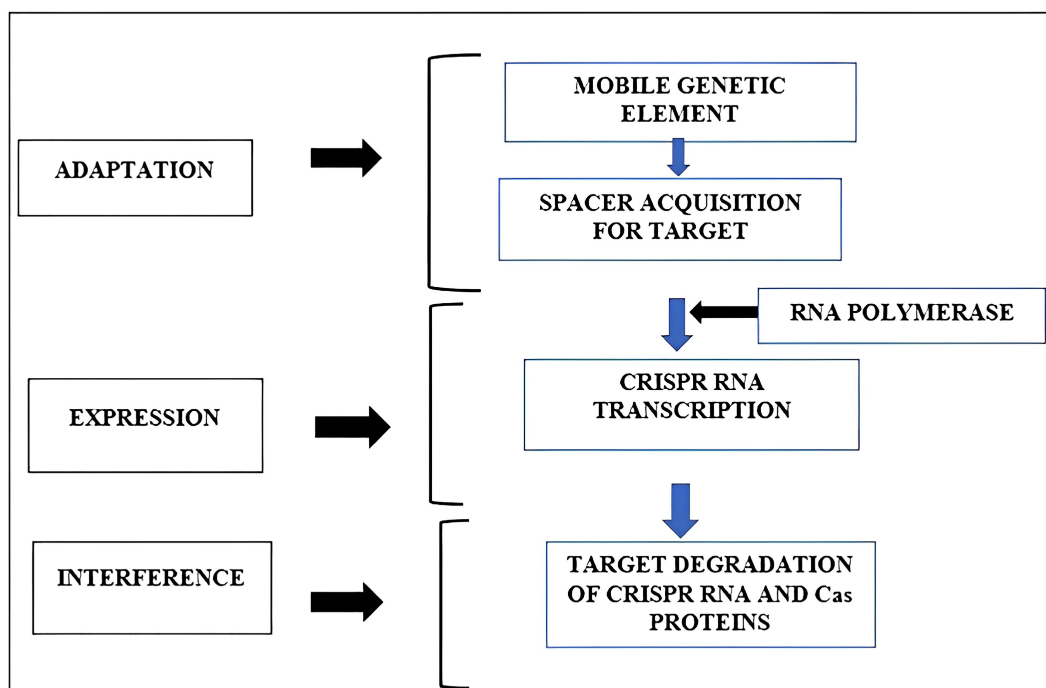

Fig 1: The main steps in CRISPR-CAS immunity are depicted here. 1) Adaptation: New spacers are inserted in to the CRISPR locus; 2) Expression: CRISPR locus transcription and CRISPR RNA processing; 3) Interference: CRISPR RNA and CAS protein detect and degrade mobile genetics.

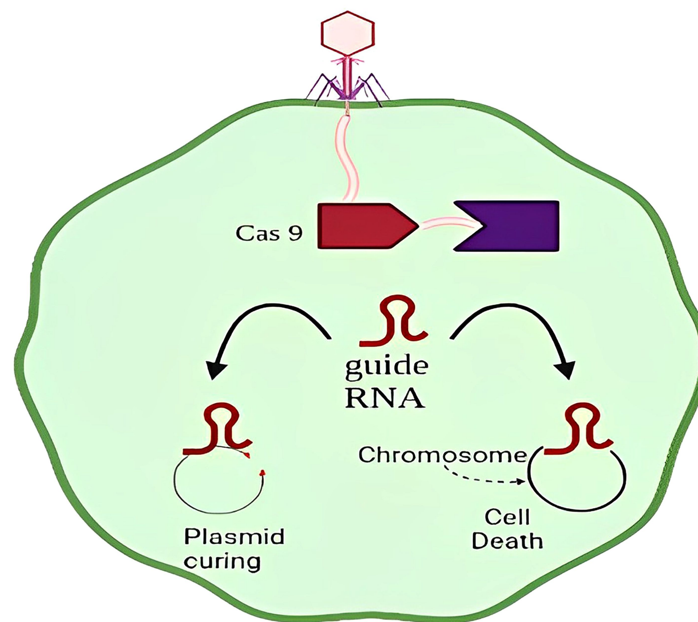

Fig 2: Use of CRISPR technique as an antimicrobial

2.1. Three different CRISPR/Cas system types

Type I, Type II, and Type III categorize the CRISPR-Cas system (as listed in Table 1). The classification was done using the distinctive genes that each type possessed. As an illustration, type I carries the cas3 gene, type II the Ccas9 gene, and class III the CSM/CMR gene. Since these two proteins are essential for the spacer, it is noteworthy that they are present in all forms and subtypes of the CRISPR system[41,42](Table 1).

2.1.1. Type I system

The majority of the organisms in this system are bacteria and archaea[43]. Six subtypes (A–F) that code for the cas3 gene. Cas344 exhibits multi-domain helicase and nuclease activity. It consists of two domains: the helicase domain DExH for double-stranded DNA cleavage and the phosphohydrolase domain HD for DNA cleavage[40, 41]. Together, these domains break down invading DNA. Each subtype of the type I system contains an abundance of Cas proteins that come together to form the complex known as the CRISPR-associated complex or the crRNA-guided surveillance complex (CAS-CADE) for defence against viral complexity. The targets complementary to the crRNA spacer can be found and attached with the help of these complexes[41]. The first instance of them was in *Escherichia coli* K12[45].

2.1.2 Type II system

The majority of this system is made up of bacteria. It is the system that connects to other CRISPR systems., This system is divided into type II-A as well as type II-B. Accordingly, the type II-A system comprises the csn2 gene, whereas the type II-B system consists of cas1, cas4, cas9 and cas2 genes. The type II system contains a specific protein called Cas9 that participates in the production of crRNA and the cleavage of foreign DNA[46]. The cas9 gene has the HNH domains and RuvC domains[47]. The HNH domain assists in DNA cleavage that complements the crRNA guide, whereas the RuvC domain is involved in non-complement strand cleavage[47,48]. The trans-activating crRNA is necessary for the type II system's crRNA synthesis (tracrRNA).

2.1.3 Type III system

Type III-A and type III-B are the two subtypes of the Type III system. The type III CRISPR-Cas system encodes for both the cas6 and cas10 genes. Endoribonuclease Cas6 performs its tasks independently of and unrelated to the CASCADE complex[49]. However, in developing crRNA and DNA cleavage, Cas10, also known as "repeat-associated mystery protein", plays a prominent role[50-51]. Foreign RNA is cut by the type III CASCADE complex facilitated by the binding to mature crRNA[51]. The bacterium *Staphylococcus epidermidis* use this process.

Table 1. The existence of different CRISPR-Cas system variants in various bacterial species

Type of CRISPR-Cas system	Associated Bacteria
Type I system[38, 52-57]	*Myxococcus Xanthus, Escherichia coli Campylobacter foetus, Bacillus halodurans, Campylobacter fetus, Propionibacterium acnes* and *Salmonella* sp.
Type II system[50, 58-64]	*Streptococcus pyogenes, Staphylococcus aureus, Listeria monocytogenes, Neisseria meningitides, Campylobacter jejuni,* and *Enterococcus faecalis.*
Type III system[65-67]	*Pyrococcus furiosus, Staphylococcus epidermidis* and *Mycobacterium tuberculosis.*

3. CRISPR-CAS SYSTEM DELIVERY

For the CRISPR system to work and deliver its genes inside a cell's genome, the ribonucleoprotein complex, which includes the key components endonuclease Cas9 and guide RNA, must be present in the cell's nucleus. However, numerous significant obstacles have been in delivering the CRISPR/Cas9 framework. The CRISPR/Cas9 device's DNA, mRNA, and protein forms often have vast diameters, making it challenging to load them onto delivery vehicles. Additionally,

the target cells must gather in the target organs or tissues, internalize, and then function as a gene-editing tool in the cell nucleus when the CRISPR/Cas9 technique is used in vivo. Since effective delivery systems for the CRISPR/Cas9 system are crucial for its clinical uses, they have recently gained research attention and developed quickly [68]. Through skipping the demands for transcription and translation, the method of delivering protein-format Cas9 nucleases offers the most fleeting expression time and permits quick editing of genes. Furthermore, the payload endonuclease Cas9 should be protected due to ribonucleoprotein complex delivery, and RNA should be pointed away from probable breakdown pathways[69]. Here, we summarise current advancements in the realm of CRISPR/Cas9 system delivery vehicles and their underlying mechanisms of action.

3.1. Factors influencing the effectiveness of the CRISPR/Cas system delivery[70]

The site-specific gene editing potential of the CRISPR/Cas system has been demonstrated in several additional applications. However, its efficacy is governed by several variable elements that must be considered, mainly if utilized for in vivo human gene therapy. These elements consist of:

1. Choosing the target DNA's site.
2. Designing of sgRNA.
3. Cutting off-target.

3.2. Phage-based CRISPR-CAS delivery system

Bacteriophages (phages), the most prevalent and extensively dispersed microorganism on Earth, provide a limitless resource for investigating the development of natural remedies. Bacteriophages are viruses only found in bacteria and can infect and kill bacteria directly. Phage therapy uses phages to combat bacterial infections and infectious disorders. Phagemids and modified temperate/virulent phages are phage vectors that introduce CRISPR-Cas systems into bacteria (Table 2)[71]. CRISPR-Cas phagemid vectors are made up of the necessary CRISPR-Cas components and a plasmid structure that has been cloned into a DNA phage-packaging sequence. Phage genome-produced CRISPR-Cas9 complex, which binds specifically to the target region and causes a double-strand DNA break during phage infection, makes up CRISPR-Cas-based Phage engineering[72-73]. The donor's plasmid had the mutations added to it. The DNA break can be repaired by recombining with the donor to produce interest mutants.

Table 2: Significant Phage-based CRISPR/Cas clinical trials

Trial strategy	Targeted/inhibited gene or strain	Type of CRISPR-Cas system used	Targeted virulent pathogen
An *Escherichia coli* is created to determine chosen phage gene deletions[74].	Non-essential gene, gene 1.77	I	Phage f1
Using a single plasmid containing donor DNA and CRISPR-Cas components, the phage genome was cleaved by CRISPR-Cas, reconstituted by homologous donor DNA recombination, and transformed into recombinant phages with deletion/ insertion mutations[75].	vpsR gene	I	*Vibrio cholerae*
Phage editing was used to create a reliable platform for genome engineering[76].	DGCC7710-pRS91R strain	II	*Streptococcus thermophiles*
Trans-activating crRNA, Cas9, and the other two system components were cloned into a single plasmid with crRNA. The CRISPR-Cas9 complex is generated once they are transformed into host cells, become expressed, and form a double-strand break by attaching to the target site. As a result, the biofilm formation is neutralised[77].	Listeria phage A511 strain.	II	*Listeria monocytogenes*
The transcribed crRNA from an external plasmid is used in addition to the native endogenous CRISPR-Cas system to complete the process. The same plasmid was used to clone donor DNA as well. This finally leads to the mutation of the genes in charge of biofilm development, producing offspring with desirable mutations[78].	LAM104 strain	III	*Staphylococcus epidermidis*
The biofilm-forming genes were inhibited by creating silent mutations at numerous genomic loci[79].	fnbA gene	III	*Staphylococcus aureus*

3.3. Advantages of phage-based CRISPR-Cas delivery system

- The delivery rate is very successful.
- The expression of transgenes is very reliable.
- Effective transfection rate.
- Capable of both in vitro as well as in vivo delivery.

3.4. Shortcomings of phage-based CRISPR-Cas delivery system

- Poor transmission efficiency.
- We have a limited host population.
- For the potential transfer of virulence genes, generalized transduction is necessary.
- Limited efficiency.

4. CONJUGATIVE CRISPR-CAS DELIVERY SYSTEM

The modified CRISPR/Cas 9 system is transmitted among bacteria through conjugation using a host-independent conjugative plasmid to delete virulence genes. The most significant impact on pathogen suppression comes through

bacterial conjugation[80]. So, bacterial conjugation is the optimum method for introducing the CRISPR/Cas 9 system into the natural environment. It is known that conjugative plasmids contain genes that make it easier for biofilms to form[81], potentially due to improved cell-to-cell contact[82], which speeds up conjugative plasmid transfer. To modify the makeup of human microbial communities, conjugative plasmids may be well suited for delivering molecular tools, many of which exist as biofilms[83–86]. Conjugative plasmids are a crucial mechanism for CRISPR nuclease delivery to bacteria. In a cis-conjugative system, the plasmid encodes the conjugative apparatus and the CRISPR nuclease [87]. A Bacterium that acquires the cis-conjugative plasmid develops into prospective donors for more Bacteria that receive the cis-conjugative plasmid become potential donors for more conjugation cycles in the future, leading to an exponential rise in the population of conjugative donor bacteria. The cis-conjugative plasmid is tested in a two-species co-culture system to find a high frequency of conjugate plasmid transfer from species to species under favourable cell-to-cell conditions. This, in turn, emphasizes the potential of conjugative administration of the CRISPR nucleases as a powerful tool for microbiome alteration[88]. The related clinical trials are listed in (Table3).

Table 3. Significant conjugative CRISPR/CAS clinical trials

Trial strategy	Targeted strain/ gene	Type of CRISPR-Cas system used	Targeted virulent pathogen
Plasmid interference and conjugation efficiency studies using *E. coli* as the heterologous host[89].	R20291	I	*Clostridium difficile*
The factors of genotypic and phenotypic pathogenicity were evaluated. The prophage integrates the plasmid[90].	EFA A, esp, cyl A	Collaboration of I, II, and III	*Enterococcus faecalis*
CRISPR nuclease and conjugative machinery encode through a cis-conjugative system in the plasmid. They developed 65 sgRNA in all[91].	Four genes with ambiguous phenotypes, 23 non-essential genes, as well as 38 essential genes.	I	*Escherichia coli*
The broad-host-range conjugative plasmid pKJK5[92] expresses cas9 protein.	aacC1 gene	I	*Escherichia coli*

4.1. Advantages of Conjugative CRISPR-Cas delivery system[93-96]

- Has a broad host range.
- The restriction-modification system cannot be used on the system.
- The system's huge coding capacity can be quickly developed.
- No cellular receptor is necessary.
- It offers a simple method for bacterial resistance.

4.2. Shortcomings of Conjugative-based CRISPR-Cas delivery system[88]

- On occasion, it could lead to a low frequency of conjugation.
- Its random insertion within the host genome is a risk factor that is connected to it.
- More significant off-target impacts were observed.

5. LIPID NANOPARTICLES/LIPOSOME-MEDIATED CRISPR-CAS DELIVERY SYSTEM

Since they have been around for a while, lipid nanoparticles have been utilized to carry nucleic acids and other compounds to cells. Lipids are also one of the elements used most frequently in non-viral gene delivery systems[97–99]. Amphiphilic molecules with hydrophobic tail and head groups make up the majority of lipid molecules. In an aquatic setting, these amphipathic lipids self-assemble to form bilayer vesicles. Thus, one or more double layers are present in the spherical phospholipid vesicles known as liposomes[100]. Because they may attach to phospholipid membranes and deliver specific components directly to the cell, they are a flexible system. Furthermore, lipid nanoparticles are used in polymeric shells by conjugating Rhamnolipid, a biosurfactant produced by *Pseudomonas aeruginosa*[101]. Increased rhamnolipid composition caused a noticeably lower level of biofilm biomass and viability[101]. Using lipid nanoparticles, CRISPR/Cas9 components can be delivered in one of two ways: either by providing genetic material (plasmid DNA or mRNA) along with Cas9 and sgRNA or by delivering Cas9: sgRNA RNP complexes. When Cas9 mRNA and sgRNA are used, the method performs similarly to microinjection[102]. However, many research teams have discovered that Cas9: sgRNA RNP complexes are extremely efficient[103-104]. Numerous studies have successfully exploited CRISPR-Cas delivery methods mediated by nanoparticles or liposomes to stop the development of biofilms (Table 4).

Table 4. Significant lipid nanoparticles/liposome-mediated CRISPR/CAS clinical trials

Nanoparticle composition	Size (µm)	Charge	Mechanism of action	Targeted virulent pathogen
DISPC:Chole[105]	0.2	Neutral	Bacterial membrane fusion	*Pseudomonas aeruginosa*
DSGPC:Chole[106]	0.2	Neutral	Bacterial membrane fusion	*Burkholderia cenocepacia*
DISPC:Chole:SLA[107]	0.12	Cationic	Targeted release throughout time.	*Staphylococcus aureus*
PC:Chole: SA[108]	2.9	Cationic	Targeting, continuous-release, and degradation prevention	*Streptococcus mutans*

DISPC: PPI[109]	0.1	Anionic	Appropriate biofilm targeting	*Staphylococcus epidermidis*
DISPC: PPI[110-111]	0.1-0.22	Anionic	Directly producing and discharging oxyacids and hydrogen peroxide, two anti-bacterial agents, into the biofilm.	*Streptococcus gorgonian*

DISPC: 1,2-dipalmitoyl-snglycero-3-phosphocholine; Chole: Cholesterol, DSGPC: 1,2-distearoyl-sn-glycero-3-phosphocholine; SLA: Stearylamine; PC: Phosphatidylcholine; PPI: Phosphatidylinositol.

5.1. Advantages of Lipid Nanoparticles/ Liposome Mediated CRISPR-Cas delivery system

- This system is compatible with biology.
- Since their structures contain neutrally charged surfaces, they have a long circulation time.
- It is being able to assemble itself.
- It is a flexible system since it can bind to phospholipid membranes and transfer components straight to the cell.
- The capacity to deliver high concentrations of inhibitors to cells at once.
- The liposomes are DNA-free, which helps minimize DNA toxicity while also increasing immunological responses, enabling the neutralization of biofilm-forming cells quickly.

5.2. Shortcomings of Lipid Nanoparticles/ Liposome Mediated CRISPR-Cas delivery system[70]

Taking account of internal and external barriers, the delivery of CRISPR/Cas9 using lipid nanoparticles exhibits certain serious drawbacks.

- The nanoparticle becomes enclosed in the endosome after crossing the cell's surface. The cell will then immediately drive the enclosed contents via the lysosomal pathway, rapidly degrading all the contained lysosomal contents.
- Efficiency is lower compared to other CRISPR-Cas systems.
- Suppose the complex of sgRNA and Cas9 can exit the endosome to avoid the situations above. However, in that case, it must also translocate to the nucleus, which is again unacceptable as it is a possible point of failure for the system.

6. ADAPTIVE LABORATORY EVOLUTION FOR ENHANCING PHAGE FITNESS

For phage treatment to be successful, phage stability and efficiency are crucial. Wild phages are especially vulnerable to changes in temperature, solute concentration, and UV radiation. Adapting laboratory evolution, a strategy for increasing organisms' evolutionary fitness and adaptation in novel settings, could be the straightforward solution. This approach employs mutagenesis combined with selected environments to push strains to their limits and ensure they thrive under optimal conditions for development. One of the research used adaptive laboratory evolution to increase the stability of three wild-type phages at high temperatures; these phages were Wc4 (Myoviridae), CX5 (Cytophaga), and P-PSG-11 (Podoviridae). After being stored at 37 °C for 60 days, the phages were treated at 60 °C for five cycles, and they showed increased stability when exposed to 60 °C for 1 hour. The modified Phage maintained the same lytic efficiency and infectiousness level throughout the evolution process. After sequencing their whole genomes, phages were found to have single favourable single alterations in their tail tubular proteins, which allowed them to survive at higher temperatures[112]. This finding added to the reader's understanding of how well-adapted phages stay when stored at higher temperatures. Chemically accelerated viral evolution (CAVE) is a recently developed approach for speeding up the development of certain traits in bacteriophages. CAVE employs repeated rounds of mutagenesis paired with selection criteria to guide the progression of bacteriophages toward a specified phenotype. In a nutshell, CAVE consists of i) introducing mutations across the phage genome, (ii) infecting a host to form a pool of mutant Phage, (iii) applying selection criteria, and (iv) analyzing phage variations and cycle repetition. This study also tested CAVE, which was found to be an effective method for increasing the thermal stability of T7 bacteriophages[113].

7. DIRECTED EVOLUTION TO IMPROVE PHAGE THERAPY

Directed evolution may be used to expeditiously reach a predetermined objective by simulating natural selection for genes and the proteins they encode. However, in contrast to adaptive laboratory evolution, the goal of directed laboratory evolution is to push the protein toward enhanced functionality[114]. In a study, Mycobacteriophage (ATCC® 11759B1TM) was employed in directed evolutionary research; it infects a non-pathogenic strain of Mycobacterium, M. smegmatis. Mycobacteriophage's lytic activity and infectiousness were improved by directed evolution in this work. In addition, the influence of inoculum size on phage adaptation was examined. Intriguingly, their results imply that smaller Phage inoculates, as opposed to bigger regimens, aid in obtaining higher titer, larger plaque size, and efficient lysis during evolution investigations. Furthermore, some mycobacteriophages can infect both M. smegmatis and M. tuberculosis, therefore the same research might be expanded by utilizing M. tuberculosis as a host to investigate the therapeutic potential of phage[115] further. According to the latest study conducted, to overcome the phage resistance of the E. coli B strain REL606 and improve medicinal uses, researchers recently employed laboratory evolution on bacteriophage as proof of concept. After 28 days of training, the phages suppressed the bacteria 1000 times more efficiently and for 3-8 times longer than the progenitor strain. Interestingly, the bacteria only needed to undergo a single mutation in order to become resistant to the untrained Phage. Still, the same result required numerous changes in the bacterium for phages generated in a laboratory[116]. Therefore, directed evolution is a promising technique to boost Phage's therapeutic value and specificity. The next step might be to use the evolved Phage against clinical isolates and assess the evolved Phage's therapeutic potential in vivo models[115,116].

8. STRATEGIES TO DELIVER THERAPEUTIC PHAGES

Bacteriophages offer enormous promise as anti-bacterial options in the post-antibiotic future, as demonstrated by

both in vitro and clinical research. For the therapy to be effective, the bacteriophages must be transported to the site of infection; otherwise, the treatment may be ineffective. To overcome this obstacle, a significant amount of research is devoted to the development of delivery techniques that will permit phages to reach their target location and exert their full potential. Numerous ways include encasing or trapping phages within liposomes, fibres, and hydrogels[117]. Liposomes are bilayered lipid nanostructures that are spherical and hollow, enabling them to transport aqueous liquids. Liposomal encapsulation of bacteriophages has been proven to protect the phages from the host environmental conditions, including acidic pH and degrading enzymes in the intestinal tract, respectively[118]. In one such research, Salmonella phages encapsulated by cationic liposomes were protected against pH 2.8 simulated stomach fluid. In addition, encapsulation has been demonstrated to increase the survival of bacteriophage in chicken digestive tracts[118]. In addition to its stability, liposomes' therapeutic efficacy in several in vivo models has been explored. For example, encapsulated bacteriophages are more effective than freely administered phages at defending broilers from Salmonella sp.[118]. Similarly, the therapeutic benefits of freely administered phages vs liposomal encapsulated Phages against K. pneumoniae have been evaluated using mouse models of burn wounds. Blood and other organs of mice treated with encapsulated phages exhibited a greater decrease in bacterial load than those treated with free-living phages. In addition, encapsulated phages had higher retention values and increased infection-curing specificity. In addition, phages administered through liposomal preparation prevented the mortality of mice even when the therapy was delayed for 24 hours[119]. Similar wound healing outcomes were obtained in a diabetic mouse model infected with *S. aureus*[120]. Hydrogels have been utilized to create pH-responsive surface coatings for lasting catheters, which release phages during infection. During an infection, microorganisms like Proteus mirabilis infiltrate the region and produce a biofilm, which raises the pH. The Phage is stored in a lower "reservoir layer" of the hydrogel, and when the pH is raised, this layer is stimulated, releasing the Phage. Biofilm development was shown to be postponed when phages were encased in a pH-responsive hydrogel produced from poly (methyl methacrylate-co-methacrylic acid) and tested in an in vitro bladder model[121]. Hydrogel-based pH-responsive surface coating smart systems and thermo-responsive polymer-based smart systems have been developed to combat wound infections. These thermo-responsive polymers, like those typically seen during bacterial skin infection, can withstand low temperatures without breaking down but disintegrate at higher temperatures[122].The phages might be contained in a thermoresponsive polymer and released gradually during infection. An example of a thermo-responsive polymer employed as nanospheres to confine *S. aureus* phage K and applied to a nonwoven fabric for use in adhesive bandages is a gel matrix of allylamine copolymerized with "Poly-N-isopropyl-acrylamide"[122]. The therapeutic effects of phages attached to fibres have been investigated in addition to liposomes and hydrogels. Using phages immobilized on fibres for topical delivery during wound dressing or in bandages is a straightforward yet efficient method[117]. Electrospinning is used to create the nanofibers, and the bacteriophages are mixed into the liquid polymer beforehand. Therefore, the Phage is encased in the nanofiber during manufacture, making it resistant to bacteria. Nanofibers made from polyethene glycol and polyester urea have been immobilized with commercially available phage cocktails. Phage immobilized on nanofibers showed anti-bacterial action against their respective hosts for up to 80 hours after application[123]. A separate investigation studied the loading efficiency, dispersion, and release of T7 Phage from cellulose microfibers immobilized by electrostatic interactions, non-specific adsorption, and protein–ligand binding. Electrostatic interactions resulted in 15–25% phage loadings adjusted to the baseline phage titer, but non-specific adsorption and protein–ligand interaction was insignificant. In addition, slow phage release from cellulose microfibers was demonstrated when phages were adhered to utilizing electrostatic interactions as an immobilization technique[124]. Stability is a big obstacle when encapsulating Phage on fibres. During the electrospinning procedure, the polymer and Phage are subjected to high voltage, which causes fast evaporation of water and changes in the osmotic state, resulting in drying of the Phage and decreased viability during storage. It has been shown that the inclusion of magnesium salts and excipients such as trehalose improves the Phage's survivability during the electrospinning and storage[123].

9. BIOFILM INHIBITION BY ACTINOBACTERIA

In terms of microbial diversity and abundance, actinobacteria are among the most impressive groups. Bacteria can be either anaerobic or aerobic, mobile or immobile, spore-forming or spore-free. They have a high ratio of guanine to cytosine and are Gram-positive. It was always thought that these bacteria only existed in soil, but modern research has shown that they permeate practically every biome[125]. Antimicrobial, antiviral, and anticancer compounds are all secondary metabolites produced by actinobacterial species. Seventy per cent of the known bioactive chemicals found in nature are now used in human medicine. Studies of the *Streptomyces* sp. genome have uncovered over fifty biosynthetic gene clusters, indicating the organism's potential for manufacturing new antibiotics. Although Streptomyces has received much credit for developing the antibiotics now in use, the possibility of other actinobacterial species should be considered. Bioactives have been shown to inhibit biofilm formation in several bacterial species—Casuarina spp. Root nodules in Tamil Nadu, India, are the source of the mycelium-forming actinobacterium known as *Frankia casuarinae*. At a dosage of 62.5 g/mL, the secondary metabolites isolated from *F. casuarina* suppressed biofilm development in Candida sp. by 81%; at a 125 g/mL concentration, they did the same in Pseudomonas. Against Pseudomonas and Candida sp. biofilm, the unique findings showed that *F. casuarinae* generate anti-biofilm compounds[126]. Actinobacteria are a promising approach for the discovery of novel anti-biofilm agents, as evidenced by the fact that secondary metabolites extracted from *Streptomyces californicus* ADR1 have inhibited 90 percent biofilm formation of *S. aureus* ATCC 29213 and MRSA ATCC 43300 at concentrations of 1.80 g/mL and 4.92 g/mL, respectively[127]

10. APPLICATIONS AND ADVANTAGES OF THE CRISPR/CAS SYSTEM

The CRISPR-cas9 gene controls numerous genes linked to virulence. CRISPR-Cas systems have been successfully modified to target virulence factors and antibiotic-resistant genes in bacteria. They represent an appealing option for both sequence-specific and programmable antimicrobials[128]. This system controls transcription and bacterial pathogenicity by regulating endogenous mechanisms. For example, a

possible human disease-causing pathogen, *Francisella novicida*[129], reproduces intracellularly by evading the host immune system. When propagated by phage capsids in vitro, they can successfully eradicate a target population, and when transmitted in vivo, they can diminish the colonization of a target population. Therefore, it may be possible to desensitize resistant bacteria to an antibiotic by curing plasmids that contain resistance genes. To introduce these approaches to the clinic, particular indications and treatment plans would need to be established. Based on their sequence, CRISPR-based antimicrobials have the distinct advantage of preventing the production of bacterial biofilms[128]. The Cas9 protein can induce chromosomal double-strand breaks in bacteria, leading to a failure of the replication fork. Ultimately, cell death inhibits biofilms' creation and favours the eradication of infections. This technique is practical and easy to use. This may prove effective in instances where it is advantageous to eradicate only a small group of bacteria within a population, something that would be challenging to perform using present tactics. Consequently, the CRISPR-Cas method, a recently created approach, offers a toolkit in the battle against lethal pathogenic diseases.

11. LIMITATIONS OF THE CRISPR/CAS SYSTEM

Despite the immense potential of the CRISPR/Cas9 system for gene editing, there are still some substantial challenges to be solved.

- The mRNA, plasmid, and Cas9 protein are all giant molecules with distinct charges, making it possible to package them all into a single vector. However, it is challenging to produce additional vectors as a result. Additionally, although typical vectors are designed to be positively charged, native Cas9 protein is positively charged, which prevents the electrostatic encapsulation of Cas9 protein[103].
- It has larger protein sizes, which reduces the effectiveness of its editing.
- Expensive approach.
- Takes up a lot of time.
- Resource-intensive.
- A CRISPR/Cas9 system component may cause the host's immune system to respond.
- Numerous, random mutations are induced at non-specific sites in the genome.
- This could result in off-target mutations.
- The Cas9-induced double-strand break activates DNA repair machinery, which controls the biochemical process by which DNA fragments are implanted (e.g., cDNAs). However, as inserting DNA fragments into the genome is outside the purview of the DNA repair machinery, targeted alleles typically carry out further changes such as deletions, partial or multiple targeting vector integrations, and even duplications[130-131].

12. FUTURE DIRECTIONS

Although prokaryotes are the original home of CRISPR-Cas systems, advances in CRISPR technology have primarily focused on eukaryotes. Regardless of whether the underlying objectives are to expand current applications or to introduce wholly new ones, progress in eukaryotes can serve as a model for similar advancements in prokaryotes. Before CRISPR-Cas can be utilized to target microbial communities, many challenges must be solved. To fully use this technology's potential to reduce clinical and environmental problems, choosing an effective distribution strategy will be essential. The genes' effectiveness would significantly rise if CRISPR-Cas constructs could be easily reprogrammed to target specific genes of interest. Future research is necessary to examine and improve the expansion of CRISPR-Cas in more useful microbial communities and to comprehend the dangers connected with this technology. To enable more precise alterations to the genome, new technologies are required. Eliminating random output would guarantee the technology's success and its therapeutic impact. Therefore, solving more complicated issues will need specific adjustments to how CRISPR may evolve.

13. CONCLUSION

The rapid emergence of antibiotic-resistant bacteria has made it more challenging to combat infectious diseases and develop new medications. This is frequently due to their capacity to build biofilms. The CRISPR-Cas system is acknowledged as one of the most current methods for controlling antibiotic-resistant strains since it is a bacterial adaptive immune system. The potential of CRISPR gene editing to alter any genomic sequence has created many opportunities for biological study and medical applications. Making the best delivery methods is necessary for continued advancements in gene editing. Conjugative CRISPR/Cas systems and phage-based delivery systems are potential viral delivery methods. The phage-based CRISPR/Cas technology does, however, have significant drawbacks. These limitations may be overcome by recent developments in phage genome engineering, which include expanding the host range of phages to support phage therapy and disrupting the immunodominant epitope of the phage capsid to reduce the immune response to phages and, as a result, produce precise variants against infectious diseases. The absence of insertional mistakes and the capacity to carefully control the dosage, duration, and specificity of delivery are just two of the many benefits of non-viral administration. Using liposomal delivery systems would create fresh opportunities for innovative research to curb different diseases' capacity to produce biofilms. The various examples provided in the "Delivery System" portion of this paper demonstrate the vast therapeutic potential of the CRISPR/Cas9 system. Therefore, CRISPR is, without a doubt the most recent genetic engineering technique.

14. LIST OF ABBREVIATIONS

DISPC: 1,2-dipalmitoyl-sn glycerol-3-phosphocholine; Chole: Cholesterol, DSGPC: 1,2-stearoyl-sn-glycerol-3-phosphocholine; SLA: Stearylamine; PC: Phosphatidylcholine; PPI: Phosphatidylinositol; CRISPR: Clustered and Regularly Interspersed Short Palindromic Repeats; Cas: CRISPR-associated.

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16. AUTHORS CONTRIBUTION STATEMENT

Ms Tamalika Chakraborty and Dr Sumana Chatterjee conceptualized and gathered the data about this work. Dr

Lopamudra Datta and Ms Ranjana Shaw contributed to this manuscript's writing and design.

17. CONFLICT OF INTEREST

Conflict of interest declared none.

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