Biomedicine & Pharmacotherapy 151 (2022) 113122

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Review

Phage delivered CRISPR-Cas system to combat multidrug-resistant pathogens in gut microbiome

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ARTICLE INFO

Keywords:
Gut microbiome
CRISPR-Cas system
Bacteriophages
MDR pathogen

ABSTRACT

The Host-microbiome interactions that exist inside the gut microbiota operate in a synergistic and abnormal manner. Additionally, the normal homeostasis and functioning of gut microbiota are frequently disrupted by the intervention of Multi-Drug Resistant (MDR) pathogens. CRISPR-Cas (CRISPR-associated protein with clustered regularly interspersed short palindromic repeats) recognized as a prokaryotic immune system has emerged as an effective genome-editing tool to edit and delete specific microbial genes for the expulsion of bacteria through bactericidal action. In this review, we demonstrate many functioning CRISPR-Cas systems against the anti-microbial resistance of multiple pathogens, which infiltrate the gastrointestinal tract. Moreover, we discuss the advancement in the development of a phage-delivered CRISPR-Cas system for killing a gut MDR pathogen. We also discuss a combinatorial approach to use bacteriophage as a delivery system for the CRISPR-Cas gene for targeting a pathogenic community in the gut microbiome to resensitize the drug sensitivity. Finally, we discuss engineered phage as a plausible potential option for the CRISPR-Cas system for pathogenic killing and improvement of the efficacy of the system.

1. Introduction

The gut microbiome can be referred to as the consortium of diverse bacterial colonies found in the gastrointestinal tract (GIT) which plays a synergistic role in host homeostasis, nourishment, and metabolism balance along with up-regulation of intestinal development, regeneration, and immune responses[1–3]. The microbial colonies residing in the gut can be classified into autochthonous and allochthonous bacteria. Autochthonous bacteria are generally native to the gut and provide immune-physiological functions along with pathogenic defense. Whereas allochthonous bacteria are out-sourced and express incidental pathogenicity while generating immune responses [4–6].

Despite the pathogenic defenses provided by autochthonous bacteria, certain external bacterial species (mainly allochthonous bacteria) manage to infiltrate the gut microbiome causing severe infections [7]. The gradual increase in the pathogens population disrupts the intricate

Abbreviations: DNA, Deoxyribonucleic Acid; RNA, Ribonucleic Acid; MDR, Multi-Drug Resistant; CRISPR-Cas, Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR associated proteins; GIT, Gastrointestinal Tract; ROS, Reactive Oxygen Species; ISC, Intestinal Stem Cell; AMR, Antimicrobial Resistance; CrRNA, CRISPR- Ribonucleic Acid; PAM, Proto-spacer Adjacent Motif; TLR, Toll Like Receptors; LPS, Lipopolysaccharide; OmpC, Osmoporin C; FliC, Flagellin C; FliD, Flagellin D; Cwp66, Cell wall protein 66; ermB, Erythromycin resistance gene; Tn5398, Transposon; FimH, Fimbrin D mannose specific adhesin; GFPT, Green Fluorescent Protein Targeting; CarO, Carbapenem-associated resistance protein O.

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https://doi.org/10.1016/j.biopha.2022.113122
Received 6 March 2022; Received in revised form 8 May 2022; Accepted 10 May 2022
Available online 17 May 2022

balance and homeostasis of the gut. This leads to dysbiosis altering reactive oxygen species (ROS) levels, bringing irregularities in the intestinal stem cell cycle, and mediates apoptosis. Thereby affecting the intestinal homeostasis and regeneration [8]. The factors leading to dysbiosis which induce resistance in these pathogens primarily remain incomprehensible. The factors like the prolonged and inappropriate use of similar sets of antibiotics, lifestyle, demography, and host biological processes (metabolism, immunity, ISC regeneration, and drug absorption) play an important role in their acutance of resistance leading to high antimicrobial resistance (AMR) [8,9]. Consequently, pathogenic AMR can reduce the therapeutic efficacy (sole application of antibiotics and antibiotics in combination) of conventional therapies, leading to the evolution of multi-drug resistant (MDR) pathogens [10]. Recent reports suggest that phage and additionally modified phage have failed to reciprocate against the MDR pathogens when applied in single doses [11]. Therefore, it becomes instrumental to devise novel combinational therapies for combating such MDR pathogens.

To overcome the limitation of current medicines, research has been conducted in the CRISPR-Cas (Clustered regularly interspersed short palindromic repeats - CRISPR-associated protein) system for gene-specific pathogen abrogation has been conducted [12,13]. Additionally, the CRISPR-Cas system poses an advantage over other available approaches because of the high specificity and possibilities of target-specific gene deletions [14]. The bacterial CRISPR-Cas system constitutes the prokaryotic adaptive immune system and is highly diversified (two classes, 6 types, and 33 subtypes) [15,16]. Even with such a high diversification, the basic function for all the CRISPR-Cas systems remains the same. Which includes three main steps: (a) Firstly the exogenous DNA fragments are subsumed into the CRISPR array (termed as adaptation) followed by (b) expression and maturation of the adapted CRISPR-RNA (crRNA) from the acquired spacers [15,17] and lastly, (c) the produced crRNA induces interference, where it recognizes and attaches to a complementary nucleotide sequence leading to cleavage of DNA/RNA by the Cas nuclease [16,18]. However, the complementary nucleotide sequence is flanked by a proto-spacer adjacent motif (PAM), which aids in the crRNA interference [17]. Such systems can be used to target the same bacterial genome. Previous reports suggest that the presence of self-targeting spacers of the CRISPR-Cas system aids in bacterial apoptosis when the cleaved DNA is not repaired immediately [19]. Therefore, it can be outlined that reprogramming Cas nuclease activity may assist in targeting specific bacterial populations thereby re-sensitizing or killing them, that exhibits the effective repurposing of the CRISPR-Cas system [16].

The immediate concern that arises in this context is the method of CRISPR-Cas system delivery into a targeted pathogen for elimination. Previously, several engineered vectors have been used for exogenous DNA transfer such as polymer nanocomposites, biolistic methods [20,21]. A recent development in genetics has shed light on a new type of modified phage where the CRISPR-Cas system can be incorporated into the bacteriophage genome [22]. This constitutes CRISPR- DNA packed in plasmid constructs within a DNA-phage system which is termed as CRISPR-Phage or CR-phage (Fig. 1) (CRISPR enhanced phage are termed as CR-phage from hereon). This novel system will potentially act based on phage adhesion on the targeted pathogen through receptors like LPS, protein porin OmpC, flagellin, pili (gram-negative), peptidoglycan, teichoic acids/teichoic acid - peptidoglycan polymer (gram-positive) leading to delivery of the CRISPR-conjugated material into the pathogen via phage [20,23]. Following this, exogenous Cas enzymes enter the targeted pathogenic system and reciprocate genetic changes as previously mentioned, leading to the defective genetic constitution that reduces bacterial resistance [24]. The admirable trait that lies in this system lies in the fact that various phage cocktails maybe be used for multiple doses to completely target pathogenic colonies, which previously were unattainable [25]. Therefore, the novelty of this system mostly relies on the fact that it incorporates both the phage; specificity with CRISPR-based pathogen knockout, which was lacking in previously investigated single system-based treatments [15]. In addition, extrinsic factors like host range, phage pharmacodynamics, and phage resistance can be modulated for enhanced effectiveness of the system [16]. Furthermore, this system effectively modulates the targeted gene expression and immuno-dynamics in the host-pathogen relationship, thereby bringing in perfect cooperation and allowing effective resistance

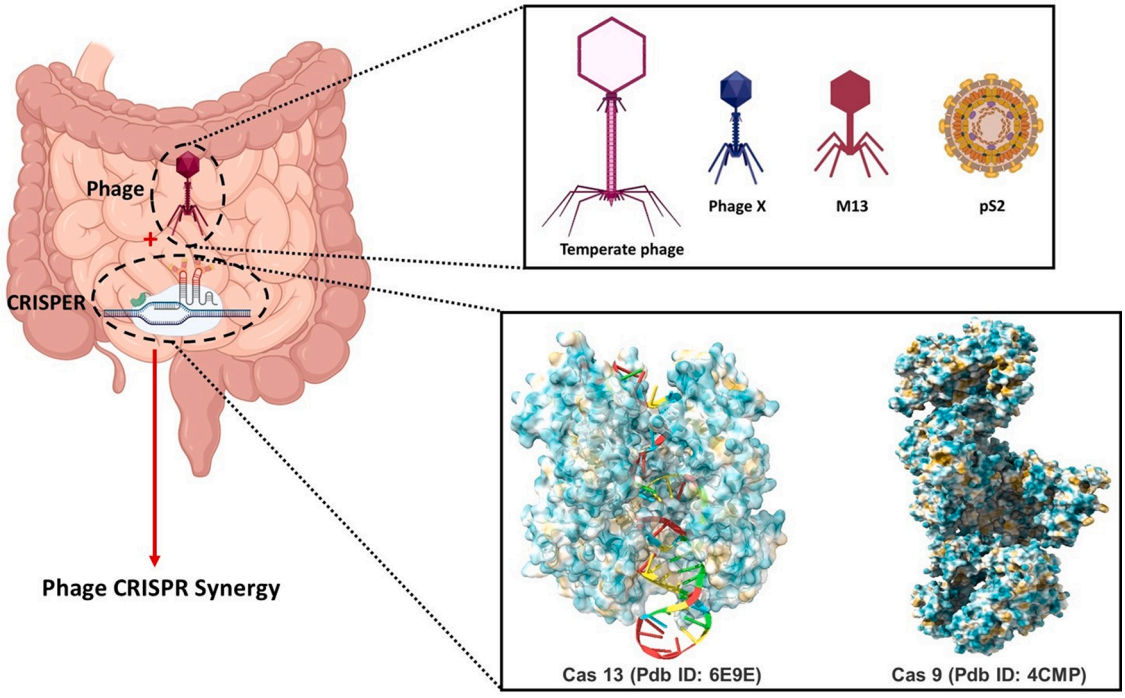

Fig. 1. Phage delivered CRISPR-Cas system in intestinal niche. Several types of CRISPR-Cas systems can be conjugated with different models of phages to form the novel engineered phage termed as CR-Phage. Cr-Phage is highly specific and can deliver CRISPR-Cas systems directly into the targeted pathogens, thereby bringing in gene deletions supplemented with phage therapy that brings loss in targeted pathogenicity in the intestinal niche.

abrogatio n [26]. Thus, developing CR-phages in a pathogen-specific manner will allow combating the ever-increasing resistance in MDR pathogens that will be utilized to target specific phage delivery along and combine CRISPR-Cas mechanism against specific resistance genes in pathogens to bring complete clearance in bacterial colonies colonizing the gut. This review focuses on the new mechanism of CR-Phages synthesis and its function against gut-specific MDR infections, with the goal of elucidating its therapeutic implications and paving the road for realistic resistance-free redemption.

2. Cr-Phage: a promising gene-editing tool

CRISPR-Cas system forms an excellent gene manipulation tool and can be used to negate excessive pathogenic accumulation through specific genomic deletions, though the major challenge lies in its delivery [27,28]. Phage are natural predators of any bacterial system but can act as an excellent vector for the CRISPR-Cas system by delivering the exogenous CRISPR-DNA, thereby solving the problem of delivery [29]. Recent insights into gut microbiome editing through strain-specific depletion have proven to be an important proof of principle in GI residual strain-specific pathogenic targeting [30]. Phage attachment is achieved by initiating the replication cycle of the phage where they bind to specific bacterial receptors (LPS, OmpC, flagellin, peptidoglycan, and teichoic acids) leading to permanent phage adhesion and infection [31, 32]. This process leads to pathogenic apoptosis via an abortive infection system (Abi) as a response to phage infection [16,33]. In addition, when engineered phage models carrying CRISPR-Cas systems, deliver to the bacterial cell, it brings additional specificity and certainty in pathogenic-gene abrogation and bacterial apoptosis [29]. Furthermore, such engineered phage mainly use CRISPR-assisted non-homologous end joining (CA-NHEJ) repair systems with homology-directed repair (HDR) at times inside bacterial genome system (Fig. 2), ensuring efficient killing in tandem with the conventional Abi mechanism [34]. Phage-CRISPR incorporation requires extensive knowledge of phage engineering and recent investigations have shown that such phage engineering and functioning is possible, even though the major challenge in phage engineering lies in preventing excessive viral replication and assembly that might affect CRISPR-Cas efficacy significantly (Fig. 2) [35].

The CRISPR-Cas system is commonly found in the genomes of gut residual MDR-pathogens and can target the bacterial system in which it is expressed [36]. Self-targeting spacers found within bacteria have been proven in studies to cause bacterial death if the bacterial genomic DNA is cleaved and not repaired [37]. Wu et al. in 2021, reviewed and depicted that CRISPR-Cas systems can be employed in bacterial detection systems, DNA/RNA conjugated targeting, and whole bacterial system regeneration [37]. Such a phenomenon emphasizes the importance of CRISPR-Cas as an antimicrobial agent. However, such engineered Cr-phage-based delivery is not available for all gut-specific pathogen genomes. Thus, functional carry-over remains an area of further research that will incite phage engineering and CR-Phage mechanisms [38].

The delivery of the CRISPR system by phage so far is dependent on either phagemid or engineered replicative phage (temperate/virulent) (Figure3). In the case of the CRISPR-phagemid system, it constitutes a DNA packed in a plasmid construct with a DNA-phage system to which CRISPR is cloned [20]. Phagemid DNA replicates inside the bacteria and is packaged in a phage capsid to be delivered via transduction to the targeted bacterial strain [39]. The mechanism of CRISPR-phagemid involves the adsorption to the bacterium by the phage particle and consequently its injection into the plasmid of the cell, leading to the expression of exogenous Cas protein and the maturation of spacers present inside the plasmid CRISPR-array into crRNA [20]. The crRNA along with gRNA guides the targeted Cas proteins to cut the proto-spacer of the bacterial genome accounts for the death of the bacteria unless it repairs its genome [16]. In addition, the efficiency of the phagemid system can be enhanced by the use of helper plasmids and facilitate the overcoming of the limitations set by the phagemid-based vector (Fig. 2) [40]. Advanced engineering allows the negation of possible backfiring of such models and pushes forward the efficacy of these models.

In the case of the CRISPR-temperate phage system, the mechanism remains the same as that of the CRISPR-Phagemid system, except of their independent viral replicative mechanism allows the phage to complete its replicative cycle [16,41]. Thereby, assembling new viral particles and

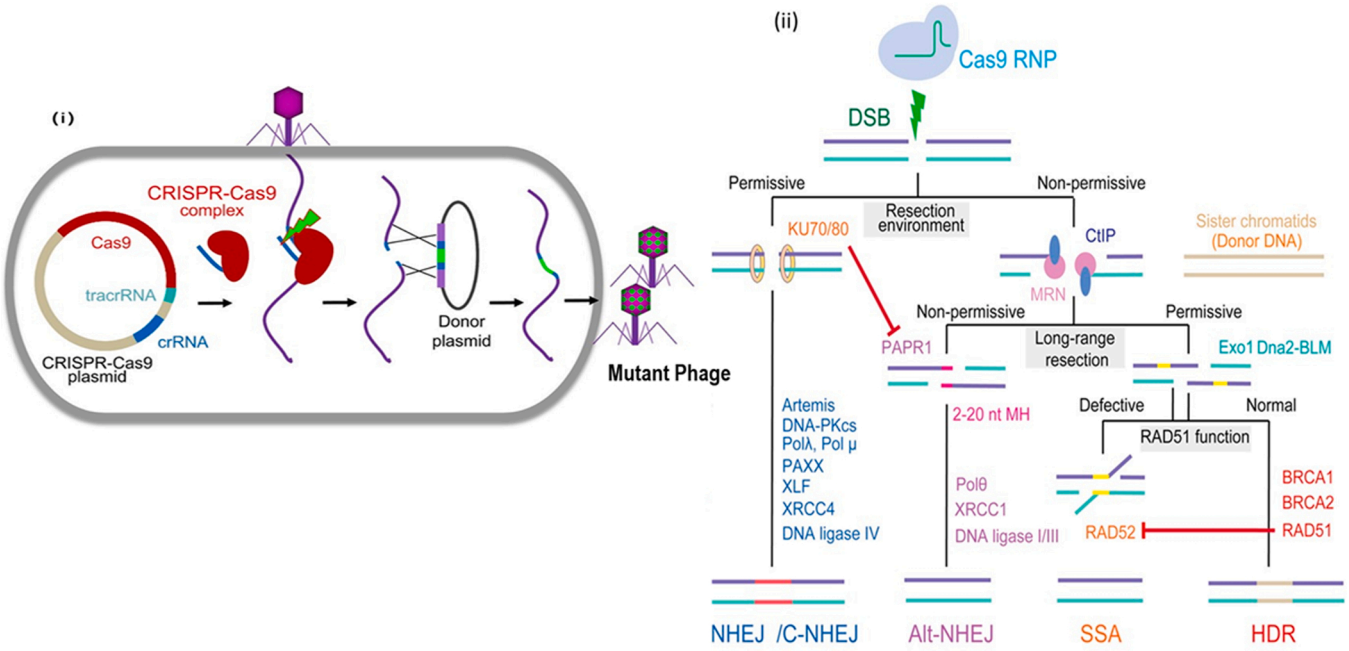

Fig. 2. CRISPR-modifiedphage (Cr-Phage). (i) During phage infection, theCRISPR-Cas9 complex is formed and binds to the target site in the phage genome,causing a double-strand DNA break. The mutations were put into the donor'splasmid. Recombination with the donor has the potential to repair the DNAbreak, resulting in mutants of interest. Adapted with permission from openaccess [36]. (ii) TheCRISPR-Cas system's NHEJ-mediated gene-editing process. Adapted with permission from open access [37].

new virions to be emancipated in the surrounding [16,41]. The CRISPR-Cas system alters MDR pathogens by altering their genetic constitution after delivery of the exogenous CRISPR-Cas system by either phagemid/temperate replicative phage (Fig. 3) [15]. Gene deletion, stop codon insertion and selected mutagenesis either result in antibiotic re-sensitization or a reduction in pathogenic burden in the gut [42]. Thus, emphasizing the critical role of phage-mediated CRISPR-Cas delivery in the gut microbial community [43]. Therefore, it can be said that phage delivery of CRISPR-Cas to specific gut-specific pathogenic islands remains a unique field of study. It provides ample capacity for further research and development, while any present obstacles can be easily reverse-engineered to find solutions, thereby enhancing the efficacy of the system. Fig. 4.

3. Phage delivered CRISPR-Cas (Cr-Phage) therapies against gut pathogens

Though there are several instances where stability hampers during pathogenic interference, the gut maintains homeostasis in normal times. The primary cause for such occurrences might be weak host immunity, robust bacterial virulence, or even pathogenic displacement in the host body [44–46]. Taking into consideration of previous advantages, phage-delivered CRISPR-Cas therapies against such pathogens have been discussed as reliable therapeutics against such pathogenic influence.

Clostridium difficile (C. difficile) (phylum *Firmicutes*) is the gram-positive anaerobic, spore-forming bacterium that was first discovered in the neonatal gut and has been a constant threat to human health for a significant amount of time with constant increment of resistance [47,48]. In general, several clusters co-exist in the Clostridium group, where a few are beneficial while the rest are pathogenic, whereby; *C. difficile* from cluster XI mainly has pathogenic roles in the human gut [49]. Several studies outline that virulent strains of *C. difficile* have antibiotic-resistant properties and infrequent exposure to antibiotics such as vancomycin, erythromycin, metronidazole [50], fidaxomicin [51], and fluoroquinolones have converted them into AMR pathogen [52]. Moreover, the AMR trait in *C. difficile* can be focused on the presence of well-known virulence genes [53], such as the *CFR*[54], adhesion proteins like Cwp66 (GroEL heat-shock protein), flagella proteins like FliC (flagellin) and FliD (flagellar cap protein) [55–57]. The same plays a crucial role in pathogenesis along with over usage of the previously mentioned antibiotics leading to the development of the MDR trait in *Clostridium,* which advocates for finding a plausible cure via the CR-phage system.

CRISPR-Cas system can be used as a potential antimicrobial against *C. difficile*, and its previous application has led to reduced expression of secondary bile acids, dipeptides, and glucose in the gut microbiome niche, showing reduced *C. difficile* growth, thereby bringing out CRISPR-Cas' role as a potential antimicrobial [58,59]. In a study, the CRISPR Cas I-B system-induced selective mutagenesis against *C. difficile* Stickland metabolism dependent selenoprotein production [60]. Its synthesis was inhibited by TargeTron insertion via the CRISPR Cas system, resulting in a reduction in the MDR property of *C. difficile* leading to an effective antimicrobial effect in the gut [60]. TargeTron insertion allowed negation of selenophosphate synthetase (SelD) expression, important for growth in *C. difficile*, faced global eradication, thereby bringing out the role of CRISPR systems [60]. In addition, the CRISPR-Cas-9 system can be used for modifying *Clostridium sp.* genome and convert it into erythromycin sensitive *C. difficile* by deletion of the ermB genes present on Tn5398 [61]. Moreover, recent investigations have used temperate

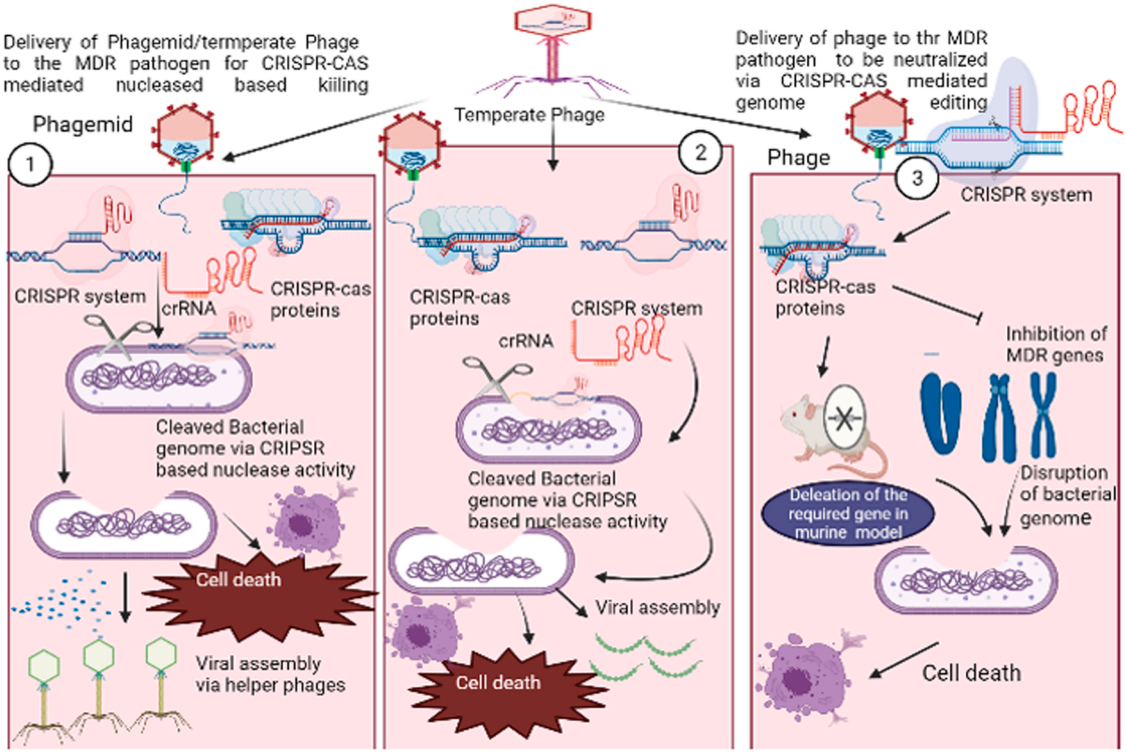

Fig. 3. Mechanism of CR-phage System to Combat the Gut Microbiome Pathogens. (1) Exogenous CRISPR-Cas DNA delivery to the bacterial genome through a phagemid in a bacterial endogenous CRISPR system. The integration of CRISPR-Cas into the bacterial genome and subsequent delivery of CRISPR-Cas results in the bacterial DNA being lysed in a crRNA-dependent manner, resulting in its death. The helper phage contributes to the phagemid's viral assembly. (2) Delivery of exogenous DNA to the bacterial genome by replication or temperate-based delivery in the bacterial endogenous CRISPR system. After integration into the bacterial genome and subsequent delivery of CRISPR-Cas, the bacterial DNA is lysed in a crRNA-dependent manner, resulting in bacterial clearance and death. The phage completes its replication cycle, assembles new viral particles, and lyses the cell, releasing newly replicated viruses into the surrounding environment. (3) Phage-based delivery of exogenous CRISPR system where it either deletes or inactivates the MDR genes of the pathogens of the gut.

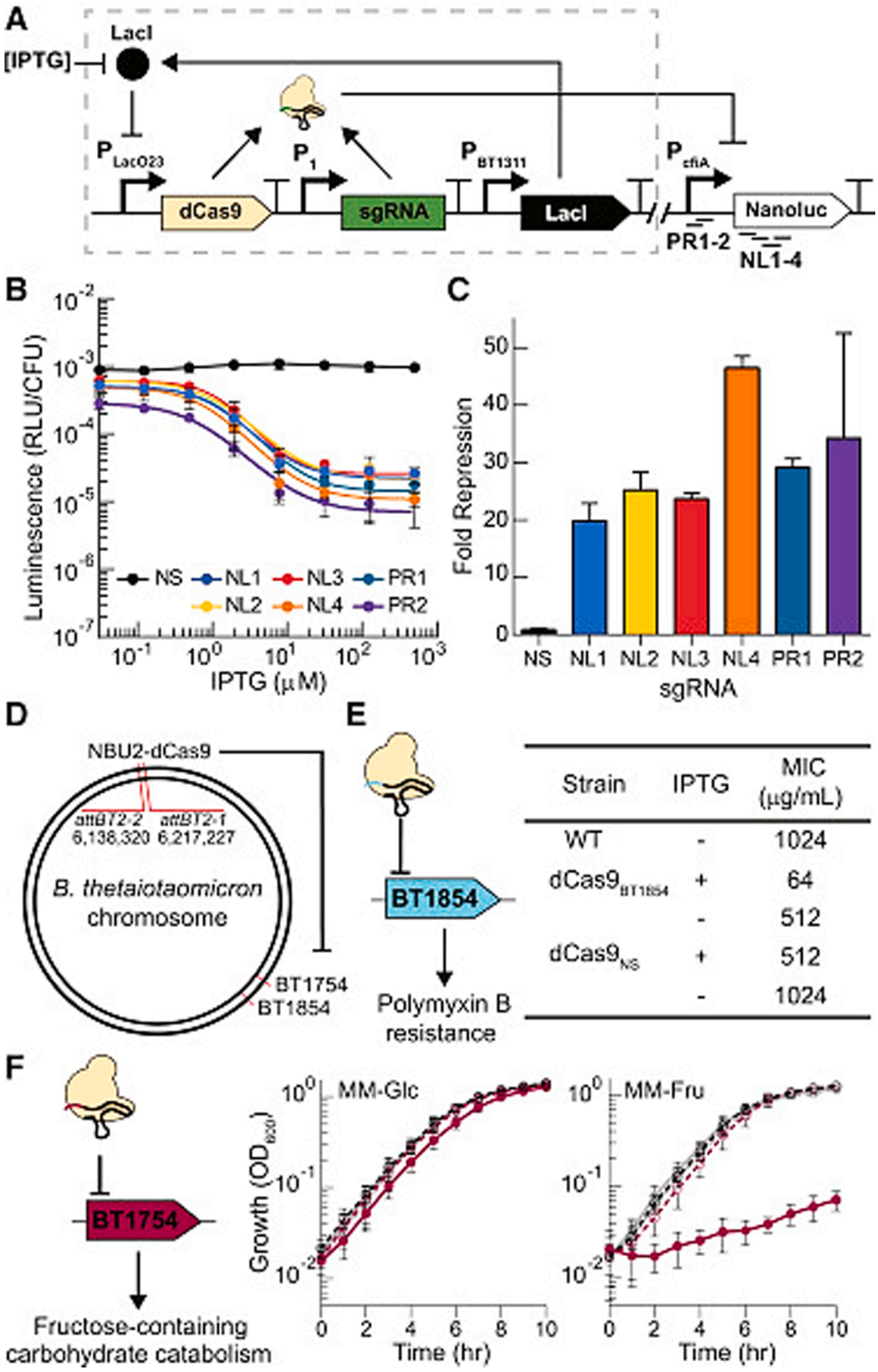

Fig. 4. *B. thetaiotaomicron* pathogenicity can be modulated by phage-delivered CRISPR. (A) Schematic of NanoLuc suppression using dCas9. IPTG stimulates the production of dCas9, which forms a complex with constitutively produced sgRNA that targets the NanoLuc (NL1–4) coding sequence or the P_{cfiA} promoter (PR1–2). The NanoLuc cassette and the IPTG-inducible CRISPRi system are separated by the plasmid backbone. (B) dCas9-mediated targeting of the NanoLuc coding sequence (NL1–4), the promoter (PR1–2), or a nonsense sequence (NS). IPTG was added to cultures in four-fold serial dilutions commencing at 500 M or no inducer. A Hill function was used to fit the response curves (solid lines). (C) Fold repression is triggered by different gRNAs in the presence of an inducer (500 mM). The color of the bars corresponds to the color of the bars (B). (D) Genomic location of CRISPRi-targeted endogenous genes. (E) Polymyxin B minimum inhibitory concentrations (MICs) for cells with CRISPRi targeting BT1854 (dCas9BT1854) against wild-type (WT) cells or non-specific control cells (dCas9NS). The reported data are the average of three biological replicates performed on three different days. (F) CRISPRi was used to target BT1754 (dCas9BT1754). WT (black), dCas9BT1754 (pink), and dCas9NS (gray) cells grow in a minimal medium supplemented with 0.5% glucose (MM-Glc) or 0.5% fructose (MM-Fru) in the presence (full line) or absence (dotted line) of 100 mM IPTG. The error bars show the standard deviation of three biological replicates performed on different days. Adapted with open access permission [106].

phage ϕCD24–2 which has been genetically engineered to express a CRISPR array composed of a repeat-spacer-repeat sequence against a conserved *C. difficile* type I-B system [62]. The delivery of CRISPR-Cas3 into the microbe cell by this designed temperate phage transduced CRISPR-Cas3 into the microbe cell during infection and expressed it together with the bacteriophage's lytic genes. The same method proved efficient at neutralizing and modifying antimicrobial-resistant *C. difficile* in the murine model and was significantly more effective than wild-type temperate phage delivery, highlighting the efficacy of CRISPR-engineered phage [62]. Bacterial clearance occurred via two distinct mechanisms: (1) irreversible genome damage caused by indigenous type I-B Cas effector proteins regulated by CrRNA, and (2) holins and endolysin release during phage replication's lytic phase [62]. This has been demonstrated in both *in vitro* and *in vivo* experimental settings, highlighting CR-phage as an effective weapon for combating *C. difficile* proliferation and resistance. Thus, phage-delivered CRISPR-Cas has emerged as an excellent genetic-engineered based antimicrobial that has immense potential to counter the rising resistance to *C. difficile*

infections in the gut. Although, a beautiful technology, further improvements are necessary. Focus can be drawn on the development of crRNA arrays against multiple repeat tandem gene structures that will negate genetic recombination and spacer excision. Furthermore, attention can be drawn to identifying and abolishing putative phage lysogenic genes promoting lysogeny. Lastly, further studies on specific CRISPR-cassette identification and engineering against *C. difficile* along with phage modification will elucidate the overall mechanism of phage-delivered CRISPR therapy. These new directions suggest heavily that the CRISPR-Cas system in association with phage can be an effective therapeutic against MDR *C. difficile* infecting the gut microbiota.

Klebsiella pnueumoniae (*K. pnueumoniae*) in recent times has gained notoriety as an infectious pathogen with hypervirulent antibiotic resistance strain with few available treatments [63,64]. The gram-negative, encapsulated bacterial species *K. pnueumoniae* belongs to the *Enterobacteriaceae* family [63]. *K. pnueumoniae* possesses hydrolyzing property via chromosomal β-Lactamase SHV-1 of β-lactam antibiotics along with a lipopolysaccharide capsule which provides *cps*-dependent resistance against phagocytosis [65,66]. In addition, the presence of self-protective pathogenic properties like fimbriae (KpfR expression) and capsule forming capabilities (via Group I or Wzx/Wzy pathway) helps in superior MDR character build-up in *K. pnueumoniae* [67–69]. In addition, extensive usage of carbapenem and other such antibiotics has led to MDR development along with biofilm formation [70]. Such properties make it hard to target and spatiotemporally negate it and therefore, most times single phage systems or antibiotics fail to bring clearance. Furthermore, studies have found the presence of type I-E and type I-E* chromosomal plasmids in *K. pnueumoniae* [71], which are responsible for providing adaptive immunity aiding in resistance build-up. The presence of type I-E CRISPR-Cas systems helps in the genetic proliferation of bla_{KPC} (bla_{KPC}-IncF plasmids^{+}) in *K. pneumoniae* populations, which remains the main cause of resistance. Due to such complexities, it becomes crucial to target such specific genes to bring sensitivity in MDR *K. pnueumoniae*, where previous therapies have failed. CRISPR-engineered phages poses the ability to combat such resistance in *K. pnueumoniae* and are paving forward as an established therapeutic in this field.

The presence of chromosomal putative type IV CRISPR-Cas system within the genome can be utilized for native CRISPR-Cas targeting and is performed by cleaving the proto-spacer sequences of their gDNA delivered by phage, thereby providing anti-microbial activity [72]. The CRISPR-Cas9 system was effective in finding the *bla(kpc2)* gene as one of the primary causes of carbapenem resistance in *K. pnueumoniae* (CRKP) strain which was achieved using a two-plasmid system of *pCasKP-pSGKP and pBECKP* (cytidine-based editing system) [73]. In addition, the same group engineered murine cytidine deaminase rAPOBEC1 with Cas9 nickase nSpCas9 to form *pBECKP* (a chimeric protein that recognized bacterial genomic sequences and can induce SSBs and convert cytidine into thymidine bases). This allowed the creation of a premature stop codon in the pathogenic FosA gene that reversed fosfomycin resistance in *K. pnueumoniae* with 100% efficiency. Both *pBECKP* and *pCasKP-pSGKP* were used to resensitize and terminate carbapenem-resistant hypermucoviscous *K. pneumoniae.* Additionally, it was shown that by utilizing CRISPR-Cas9 from Pyogenes and a small guide RNA (sgRNA), the MDR genes in *K. pnueumoniae* could be deleted via bacteriophage phiKpS2, resulting in a decreased load of *K. pnueumoniae* using the CR-phage system [74]. The reduced load of pathogenic *K. pnueumoniae* showed less pathogenicity and effectivity towards antibiotics, which made it possible to bring its clearance. Lastly, encapsulation of phage via liposomes allowed overcoming the problem of circulation and providing protection against gastric juice and host immune system [75]. Studies have found that there is an enhanced reduction in pathogenic load via encapsulated phage CRISPR system compared to non-encapsulated phage system [76]. Thus, these finding suggests that CRISPR-Cas delivery by engineered phage (Cr-Phage) is possible and is advantageous for re-sensitizing and terminating *K. pnueumoniae* in the gut. Though effective *in vitro* conditions, there has been no investigation regarding its efficacy in complex *in vivo* situations. Therefore, a large number of clinical trials are required for such systems to make it a practical therapy against *K. pnueumoniae* infections. Only future research in this field will elucidate its effectivity as a therapy against the same.

Escherichia coli (E. coli), a prominent commensal and symbiont of the gut, can sometimes pose as a revered pathogen inside the gut with an enhanced resistance profile and hiked cases in the past decade [77]. The *Escherichia coli* strain ST131 is one of the most studied pathogenic bacterial forms and has the inherent capability to modulate the effect on various antibiotics like fluoroquinolones [78], owing to its AMR property and its capability to form FimH adhesion-dependent biofilms [79]. The epidemiology-related clinical studies conducted in Iran [80], Mexico [81], and India [82] have reported the different degrees of resistance to various antibiotics including cephalosporin, SXT, ciprofloxacin [83]. *E.coli* has exacerbated the present situation of its ever-increasing resistant profile to all known major classes of drugs for which novel therapies like phage delivered CRISPR-Cas retardation is required.

To counter-resist, the MDR *E.coli* strains, the CRISPR-Cas system has surfaced as antimicrobials. It can be delivered through phage-mediated mechanisms to provide a global eradication of *E.coli* [84]. Köse et al. in 2020, showed that replacing the Cat gene against the catabolite repressor protein (CRP) gene using CT-CRISPR-Cas9 editing along with the panD gene of the bacterial chromosome resulted in decreased pathogenic load [15]. Additionally, the CRISPR-Cas system can overcome *E. coli's* chloramphenicol resistance, demonstrating the CRISPR-Cas system's antimicrobial potential. This is accomplished by regulating the *E.coli* system's metabolic pathways [85]. The same results in highly decreased pathogenic load since they are one of the fundamental components of resistance to *E.coli* [86]. Additionally, the CRISPR-Cas9 system was effectively employed in a study to eliminate and replace pathogenic genes in enterotoxin *E.coli.* (ETEC) [87]. Moreover, in another scenario, the CRISPR-Cas9 system was used in association with Cytidine deaminase (PmCDA1) to get specific point mutations (SPM) at cytosine that converted cytosine to thymine (in a five-base gap of target sequences) to inactivate the virulence of *E.coli* [88]. The mutation efficiency was boosted with DNA glycosylase inhibitor in association with a degradation tag (LVA tag) that permanently brought in mutations and destroyed *E.coli* pathogenicity, thereby bringing out the role of CRISPRs as an antimicrobial [89]. Several investigations using CRISPR as an antimicrobial can be used against luxS, β-lactamase, Stx, and LEE pathogenicity islands [21,90,91]. Thus, the CRISPR-Cas system is quite effective against several pathogenic genes and can be beneficial in curbing high incidences of pathogenicity shown in *E. coli* and can be further enhanced when target-delivered by Cr-Phage.

To bring out Cr-Phage delivered efficacy, Lam et al. used a single-stranded DNA (ssDNA) filamentous inovirus M13 phage, against gut residual Sm-resistant (Sm^{R}) *E.coli* [92]. CR-Phage selectively targeted the pathogen and depleted its virulence through the introduction of genomic deletions. Lam and colleagues were able to construct two sets of CRISPR-Cas9 vectors: 1) non-targeting (NT) and 2) GFP-targeting (GFPT) CRISPR-Cas9 using *E.coli bla* gene. They utilized the f1 origin of replication and inserted it into low-copy CRISPR vector pCas9 that produced pCas9-NT-f1A/B and pCas9-GFPT-f1A/B respectively [30]. This cassette of the engineered CRISPR-Cas system was inserted into M13 that produced two M13 phage strains namely; NT-M13 and GFPTM13 [30]. These strains were used to infect (Sm^{R}) *E.coli* consecutively followed by carbenicillin which resulted in reduced growth and lower rates of recovery. The engineered phage M13 was able to target specifically and deliver the CRISPR-Cas9 gene that induced genomic and chromosomal deletions and helped in an overall reduction of its specific growth [30]. Whole-genome sequencing of each deletion (45 bp-82.6 kb) was performed demonstrating the *E. coli*-mediated Cas9-induced double-stranded breaks through homologous

recombination, which brought out the efficacy of CRISPR-Cas9 induced restriction on resistant *E. coli* [93]. As a result, CRISPR administered by phage (M13) was extremely successful and suitable for introducing genetic deletions via conserved DNA repair mechanisms. The main advantage to this system lies in the fact that simple genetic abrogation in *E. coli* would not affect the whole gut microbiome but is efficient in clearing up specific pathogenic islands. Although elusive, this technique faces a few challenges like an escape to CRISPR-Cas9, spacer losses, and CRISPR gene deletions for which future investigations need to be conducted. It can be said that phage-delivered CRISPR-Cas (Cr-Phage) therapy in MDR *E. coli* is in a neonatal state and research and further clinical trials are required to bring practical advantages to this mechanism. Thus, Cr-Phage therapy provides a directive path to specific gene targeting in *E. coli* allowing its clearance, without hampering gut microbiota, and with futuristic approaches, it can pose as a reliable therapy.

In several instances, the presence of *Pseudomonas aeruginosa* (*P. aeruginosa*) in the gut microbiome has led to pathogen-specific infections in the gut microbiota [94]. *P. aeruginosa*, is an anaerobic gram-negative pathogen of the *Pseudomonadaceae* family known to be extensively drug-resistant and has been reported to cause infection of the intestines [95,96]. *P. aeruginosa,* too, relies on biofilm formation and quorum sensing and uses three genes like las, rhl, and quinolone signal transduction system in an acylated homoserine-dependent manner [97, 98]. These gene products and quinolone control various pathogenic factors including enzymes like elastase, protease, and other substances like pyocyanin which promote pathogenesis and infectivity [99]. General antibiotics mainly fail to penetrate the biofilm thereby, initiating the so-called multidrug resistance, thereby requiring specific gene editing mediated therapy [100,101].

Complete resistance of *P. aeruginosa* can be overcome with CRISPR-Cas delivered by a phage that targets the pathogen island with biofilm eradication, lowering antibiotic resistance, and finally killing [15]. Infection of *P. aeruginosa* strain PA14 was reduced by DMS3 engineered phage with CRISPR-Cas system when five out of six endogenous CRISPR-Cas genes were deleted [102]. The use of phage in conjunction with the CRISPR-Cas system increased the specificity, but also enhanced the biofilm penetration ability of antibiotics and phage. Once penetrated through the pathogenic biofilm islands, the engineered phage could modulate the pathogens and target them by using CRISPR-Cas nuclease activity to induce killing [102]. This was mediated mainly by lytic and lysogenic mechanisms of the DMS3 phage along with DNA denaturation and modification by the injected CRISPR-Cas system. Therefore, DMS3 engineered phage system forms an important insight where Cr-Phage was able to deliver CRISPR RNA to mediate biofilm eradication, and enhanced antibiotic penetration. Furthermore, such targeting led to phage and CRISPR combined bacterial killing that further brings out the importance of CRISPR-delivery by phages in *P. aeruginosa*. However, these findings are still in an early state and further research in gene delivery and phage-bacterial biologics has to be determined to deduce its further application in combating MDR *P. aeruginosa*.

Bacteroides thetaiotaomicron (B. thetaiotaomicron) of *Bacteroides* is a rare pathogen of the intestine. Though previous reports suggest that *Bacteroides* class are mostly present as commensals helping in the fermentation of host diet or microbial-derived polysaccharides [103]. *B. thetaiotaomicron* can act as an opportunistic pathogen in the gut, mainly due to weak host immune systems. It is known that *B. thetiaotaomicron* are gram-negative, non-spore-forming, obligately anaerobic, antibiotic-resistant bacteria that harbor in the human gut [104]. *B. thetaiotaomicron* possesses certain virulence factors like adherence proteins, penta-acylated LPS, as well as the ability to defend itself from the host immune response in oxygen-depleted circumstances and phagocytosis, resulting in increased resistance and a robust infection profile [104]. In addition, it is known that *B. thetiaotaomicron* possesses a lincomycin resistance gene (*linA$_N$)*, and transposon NBU2 may be responsible for its transmission into the Bacteroides group. NBU family is miscellaneous and all have similar integration strategies with different target sites and carries various resistance gene that might provide a broad resistance, though this requires further investigations [105].

To find a justifiable therapy for such resistance-related infections, Mimee et al., 2014 have presented a reprogrammable system that can lower the recombinant and endogenous gene expressions in *B. thetiaotaomicron* using CRISPRi (CRISPR-interference) system and recombinase system [107]. *B. thetiaotaomicron* is highly resilient and shows superior resistance in the gut microbiota [107]. To target this and reduce its resilience, the CRISPR-Cas system was designed against LpxF (provides resistance towards inflammation-associated cationic antimicrobial peptides, like polymyxin B) from gene BT1854. CRISPR-Cas9 system incorporating dCas9 (regulated by IPTG-inducible PLacO23 system) was incorporated resulting in the production of specific sgRNA against BT1854 (dCas9$_{BT1854}$) that reduced resilience and resistance towards polymyxin B [107]. *B. thetiaotaomicron* also shows robust carbohydrate metabolism important for successful establishment in the gut and its growth. BT1754 gene is a two-component detector and controls BT1757–1763/BT1765 (fructose-containing polysaccharide utilization locus), helping in its fructose metabolism and establishment. CRISPR-Cas9 engineered system was incorporated in a similar way as (dCas9$_{BT1854}$) against gene BT1754 (dCas9$_{BT1754}$) that resulting in reduced fructose metabolism while keeping glucose metabolism normal [107]. Through in-depth investigations, a plausible easy phage delivery of this mechanism has been established. A novel class of phage called crAss-like phage mainly targets *Bacteroides* species and crAss-like phages DAC15 and DAC17 have been recently reported to infect *B. thetiaotaomicron* and induce killing [108]. DAC15 and DAC17 can be similarly engineered with the CRISPR-Cas9 system as shown by Mimee et al. in *in vitro* study [107]. In the study, phage-mediated CRISPR-Cas delivery against *B. thetiaotaomicron* was made possible using *in vitro* techniques. The study enlightened the fact that CRISPR mediated interference (CRISPRi) will be possible in gut microbiota and when delivered by phage can pose as a feasible therapy for targeting *B. thetiaotaomicron*-related infections, although further research is required to bring conclusive facts.

CRISPR delivery of phages via CR-Phage is a completely novel technique and comes with the benefit of combinational therapies relating to phage and CRISPR (Fig. 5). Table 1 summarizes the clinical significance of Phage-CRISPR synergy. The enhancement in bacterial targeting, specificity, and overall efficient killing of bacterial pathogenic islands, with a global approach to curb the MDR traits of the aforementioned pathogens affecting the gut; will determine a new direction in phage and gene editing biologics (Fig. 4). In addition, when several antibiotics are failing to reciprocate regulation in the pathogenic population, such technologies in mono and combined with pre-existing antibiotics approach will further determine the MDR curbing efficacy. However, it is difficult to state the overall therapeutic functionality of such systems due to many hurdles that are still untouched. Experimental obstacles like CRISPR-cassette mutations, gene deletion, pathogen mutations and escape from CRISPR targeting remain to be investigated and a field of further research.

4. Immune system modulates the overall Cr-Phage targeting

The functionality and efficacy of Cr-Phage delivered CRISPR-Cas system depends on how efficiently the phage can target and attach to the bacterial cell wall followed by phage adhesion and delivery of the genetic material [30]. Recent investigations have exhibited that phage is mainly constituted of foreign viral particles (capsid, nuclear materials), which initiate immune system-mediated action against phage by producing anti-phage antibodies [110]. These anti-phage antibodies while targeting phage also apprentice in bacterial targeting and overall phage therapy. Hence, phage therapy can be curated by the host immune system and control the overall phage targeting efficiency. Investigations

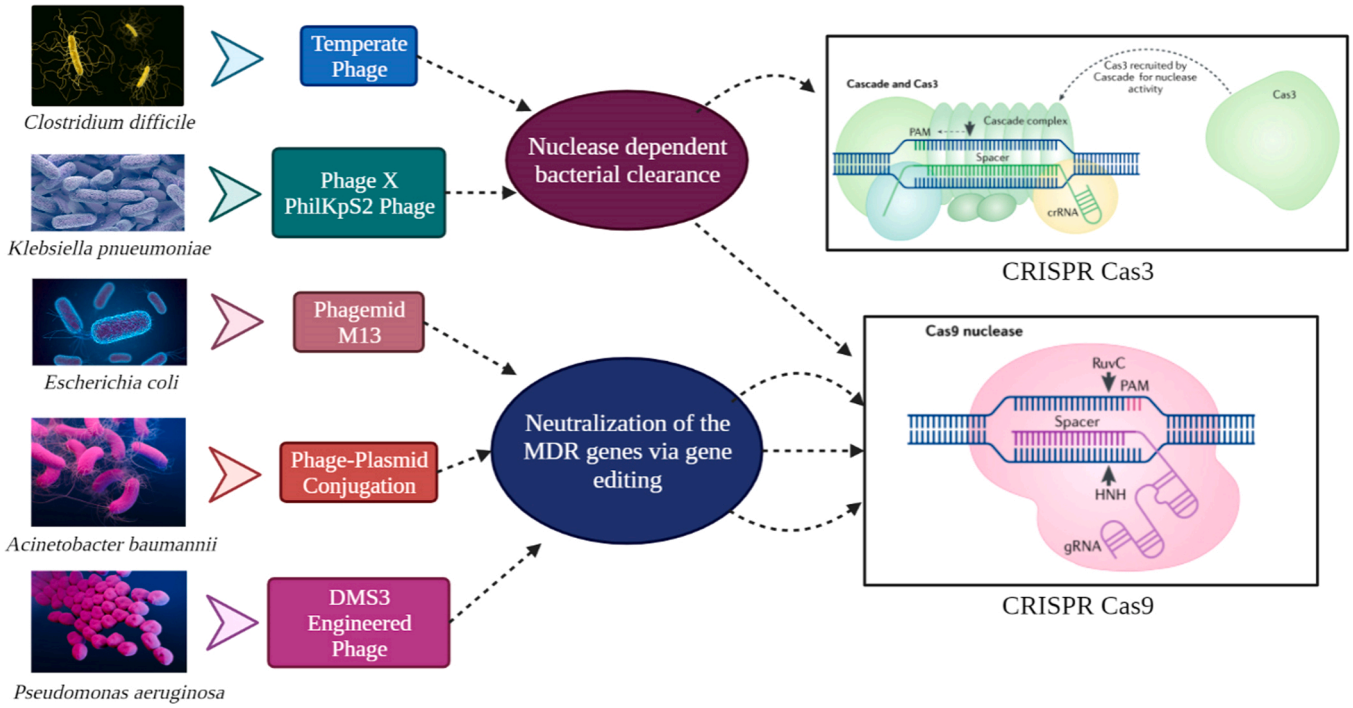

Fig. 5. CR-phage and MDR pathogen resistance in the gut microbiome. *C. difficle* utilizes temperate phage to delivery CRISPR Cas3 to induce bacterial killing. *K. Pnuemoniae* utilizes Phage X to cause bacterial clearance in a nuclease dependent manner upon delivery of CRISPR-Cas 3. Genetic editing of MDR genes upon delivery of CRISPR-Cas 9 by Phagemid, Phage-plasmid, engineered phage and M13 phage causes clearance of *E.coli* and *A. baumannii* and *P.aeruginosa*.

Table 1
CR-phage to Combat MDR Pathogen Resistance in Gut Microbiome.

Pathogen	Bacteriophage System	CRISPR-Cas System	Preclinical study	Neutralization of MDR pathogen via combinational therapy	Reference
Clostridium difficile	temperate phage	CRISPR-Cas 3	*in vivo*	nuclease dependent bacterial clearance	[62]
Klebsiella pnueumoniae	Phage X (encapsulated with mutated capsid)	CRISPR–Cas 9	*in vivo*	nuclease dependent bacterial clearance	[75]
Klebsiella pnueumoniae	PhilKpS2 phage	CRISPR-Cas 9	*in vitro*	neutralization of the MDR genes via gene editing	[74]
Escherichia coli	Phagemids M13	CRISPR-Cas9	*in vitro*	neutralization of the MDR genes via gene editing	[35]
Acinetobacter baumannii	Phage -plasmid conjugation	CRISPR-Cas9	*in vitro*	neutralization of the MDR genes via gene editing	[109]

have shown that the gut-residual phage is an important regulator of the host immune system, and forms the interface for phage-immune system interaction through transcytosis-mediated action in the inner lying epithelial cells to modulate the immune system responses [111]. The phage can also incite interaction with TLRs and successively TLR-mediated immune response against themselves [111]. The dynamic nature of the phage allows them to alter the pathogenic property of bacterial species which can be exploited to kill the pathogens [112]. Phage can stimulate enhanced bacterial phagocytosis which is beneficial to target the pathogens. The bacteriophage can also induce cytokine-mediated innate anti-inflammatory and pro-inflammatory immune responses, that aids in final bacterial clearance [111]. Therefore, it is evident that there is a very close interaction between phage and the host-immune system. Immunomodulation of CRISPR-phage is a very important aspect and is gaining importance in current scenario. Utilizing such interactions, phage delivery of CRISPR-Cas system to target pathogens can be shaped accordingly, and specificity can be upregulated accordingly, thereby directing the stem down for the rising MDR pathogens more efficiently.

5. Engineered Phage-CRISPR system: potential for combating MDR pathogens

Phages can be modified with exogenous DNA (CRISPR-Cas) to overcome resistance in several pathogens and enhance the effectiveness of the system [113]. Though efficacious, this technology might face certain problems like phage resistance, phage pharmacodynamics, and lastly host range that becomes a problem in long-term applications [114]. Therefore, recent advances have shown that phage delivery of CRISPR (Cr-Phage) can be further enhanced with specially modified/engineered phage to overcome the barriers that this therapy might face altogether [114]. Addressing such problems will lead to a better platform for this gene-editing-based therapy, and improve the efficacy of the system.

Phage resistance within the bacteria is an important aspect, which plays a pivotal role in determining the sensitivity of phage-based therapies like CR-phage [115]. CR-phage-based therapy can not only face phage resistance but also come across an overall phage-mediated bacterial infection efficiency reduction (loss of fitness) during bacterial infection. A solution for such complexities lies in combining multiple phage with engineered phage to form a phage cocktail that may help to minimize the aforementioned problems and thus, improve the therapeutic efficacy (Fig. 6) [116]. Previously, such phage cocktails have

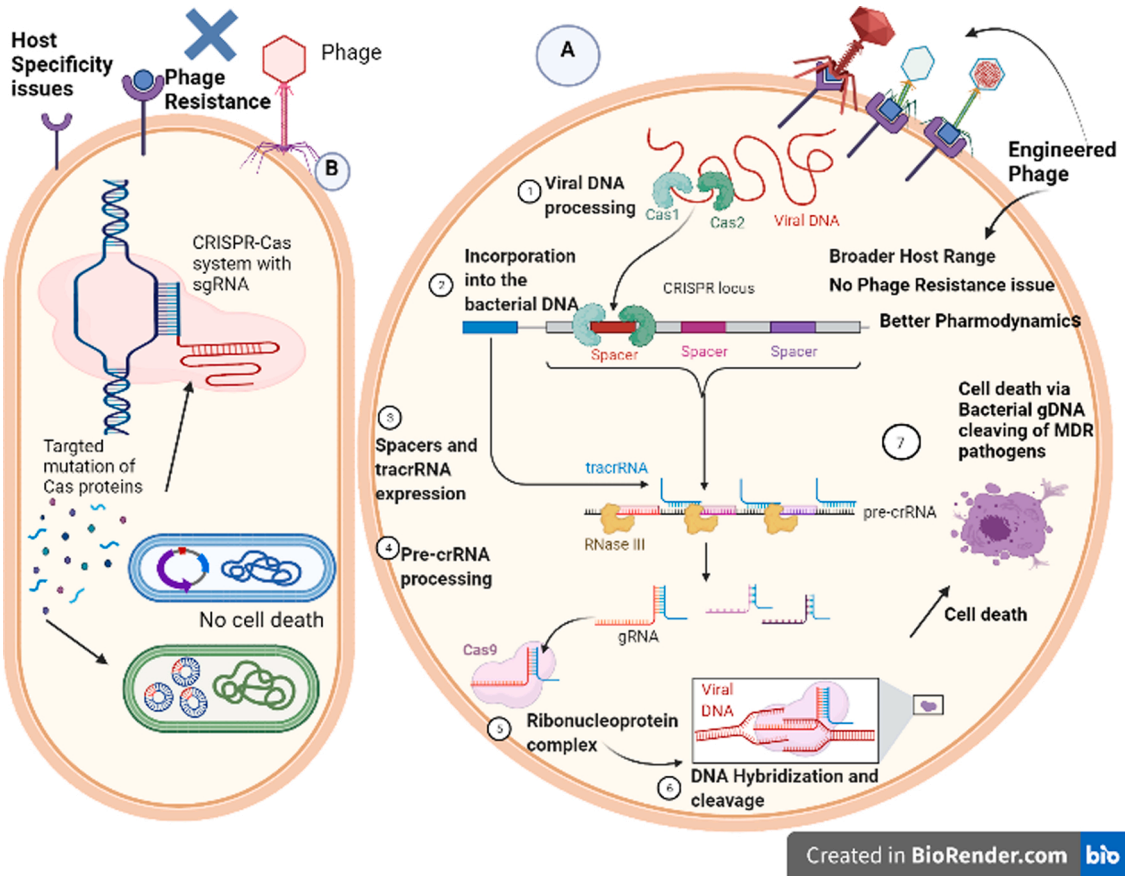

Fig. 6. Engineered-phage CRISPR combinational therapy to enhance bacterial killing.

been used and can be effective in paving the path for Cr-Phage and CRISPR-Cas delivery in gut residual MDR pathogens.

Engineered phage along with Cr-Phage capitalizes on the receptor binding mechanism of phages and by modifying such receptors, novel strategies can be developed to personalize or extend the specific host range (Fig. 6) of CR-Phage. Furthermore, such modifications can lead to better adsorption to the target pathogen. Previously, Bowers et al. in 2018 and Yehl et al.in 2019, was able to create plasmids carrying appropriate phage tail genes and a T7 phage-packaging signal that was transfected into *E. coli* to increase the receptor binding capability. Yehl et al., in 2019, further showed that phage T3 solely modified with receptor genes created via random mutagenesis resulted in a broader panel of altered phage known as phage-bodies that reduced the generation of phage-adsorption resistant bacterial colonies in the gut. Therefore, such engineering enhanced and curated the host range thus modulating the delivery aspect of the phage.

Phage pharmacokinetics is yet another important factor and has a crucial role in CRISPR-engineered phage therapeutic efficacy [117]. Without proper considerations, future therapies involving Cr-Phage may take a longer duration for effective therapy. Additionally, a certain amount of phage cocktail should reach the target bacteria when applied for required efficacy, that will lead to proper adsorption to the pathogens [118]. Phage when injected into the body faces clearance from the host body and therefore adequate attention should be provided to allow proper levels of phage to be injected for non-hampered effectivity [118]. Furthermore, encapsulating phage in alginate or employing liposomes or polyethylene glycol-based platforms have been used to increase phage adsorption, dispersion, and persistence along with delaying clearance from the body [119]. Encapsulation protects phage from stomach acidity and the immunomodulatory functions, allowing them to circulate more freely for extended durations in mammals [119]. Therefore, encapsulation of CR-Phage can enhance it effectivity in combatting gut residual pathogens. These instances confirm the utility of CRISPR-engineered modified phage over normal CRISPR encoded phage and form an insight that has resulted in abrogating phage resistance, enhancing host range and pharmacokinetics thereby concreating and filling gaps in normal phage delivery of CRISPR. CR-phage efficacy can be propagated to endless proportions leading to an effective platform that can deal with the ever-increasing resistance of pathogens infecting the gut, although severe investigations are required to make it perfect.Fig. 7.

6. Conclusion

Infection to the gut microbiome through MDR pathogens remains high and is a matter of concern at present. Failure of previous mono and combinatorial therapies (phage, antibiotics, and cocktails of both) in this arena has led to apprehensions following to the investigation of novel ways in gene-editing tools and their delivery. CRISPR-Cas mediated gene targeting and its delivery by bacteriophages (Cr-Phage system) remains to be a besuited mechanism in combating MDR pathogens affecting the GIT. Additionally, the CR-Phage-based gene-editing system has proven to be efficacious and poses an authentic therapy against MDR pathogenicity. It exploits the phage-CRISPR counter selection system and utilizes the advantages provided by both the systems in successful bacterial gene modification (virulence genes, pathogenic cassettes) leading to death (Box2). Moreover, Cr-Phages can re-induce antibiotic sensitiveness and heightened efficacy through proper targeting, penetration, and resistance breakage, which remains need of the hour. Phage biology, host transcriptome, and immunity spearhead and orchestrate the overall targeting and delivery. Bringing further curation to such significant players in this synergy will allow us to comprehend the

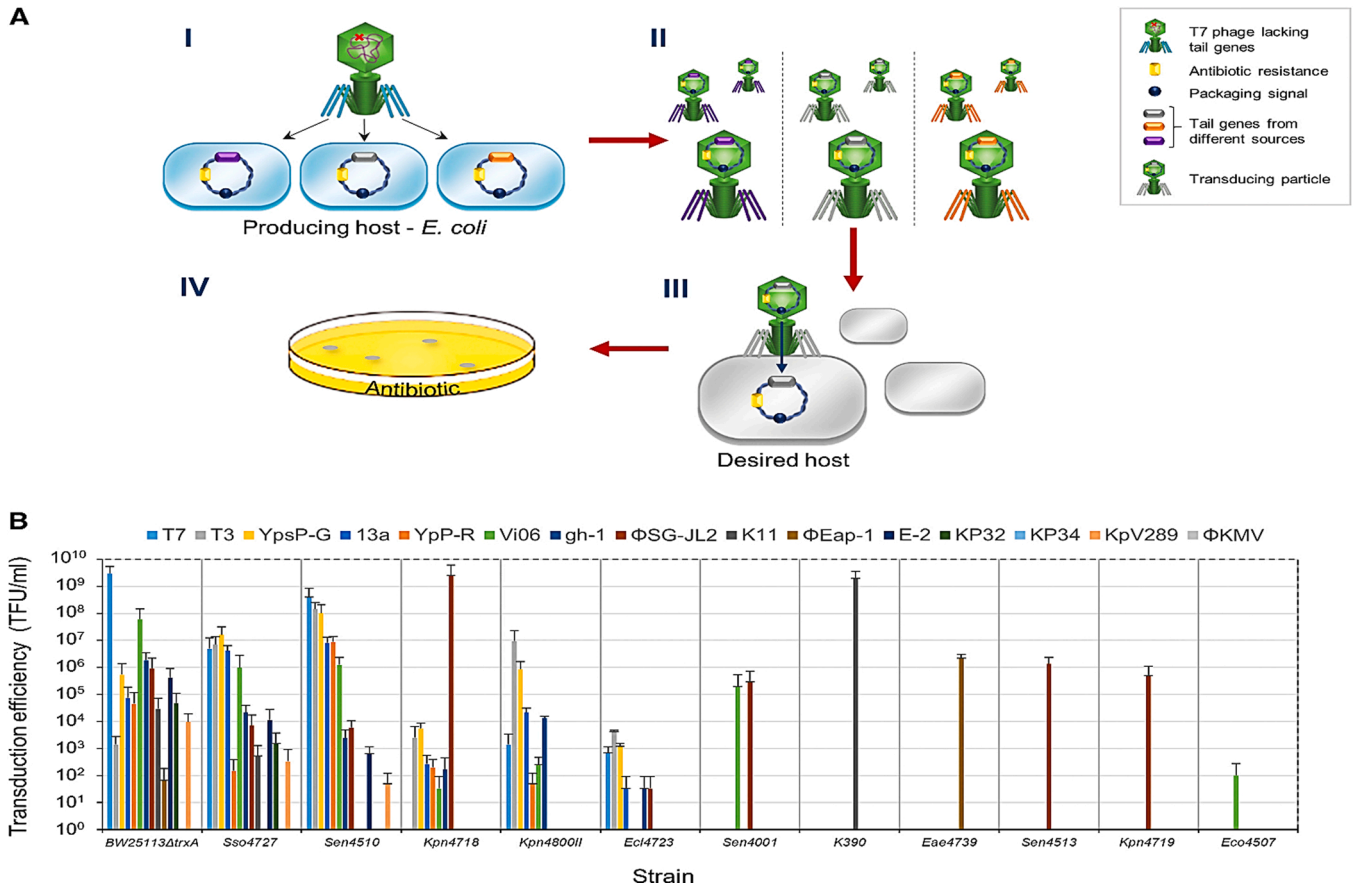

Fig. 7. Engineered T7 particleswere able to transduce novel hosts with enhanced efficacy. Adapted with open access permissionfrom [120]. (A) Schematic depiction of the procedure.(B) DNA transduction of different hosts bydifferent hybrid particles. Bars represent the average ± SD of the number oftransduced colonies per mL (TFU/mL) by the indicated hybrid particles into theaforementioned indicated host.

overall mechanism and enhance the precision of the current approach. Further investigational steps in this field can be testing of Cr-Phage technology in animal models and bringing clinical trials. Profound data sets in molecular mechanisms, gut microbiome orchestration, and phage dynamics are required for CR-phage establishment as a reliable technique. Although phage-based targeting is an old approach, the use of engineered phages (especially CR-phages) is quite novel and still in infancy. Further research will elucidate the phages delivered CRISPR-Cas system contrivance that will help us to curb MDR gut-residual pathogens.

CRediT authorship contribution statement

Arijit Nath: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft. **Rahul Bhattacharjee:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft. **Aditya Nandi:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft. **Adrija Sinha:** Methodology, Formal analysis, Investigation, Resources, Writing – review & editing. **Sulagna Kar:** Methodology, Formal analysis, Investigation, Validation **Nikita Manoharan:** Methodology, Formal analysis, Investigation, Validation. **Shirsajit Mitra:** Methodology, Formal analysis, Investigation, Validation **Abhik Mojumdar:** Methodology, Formal analysis, Investigation, Validation. **Pritam Kumar Panda:** Methodology, Investigation, Formal analysis, Software, Validation **Swadheena Patro:** Methodology, Formal analysis, Investigation, Resources, Writing – review & editing **Ateet Dutt:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing – review & editing. **Rajeev Ahuja:** Conceptualization, Methodology, Software, Validation, Resources, Data curation, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Suresh K. Verma:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision. **Mrutyunjay Suar:** Conceptualization, Methodology, Software, Validation, Resources, Data curation, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Author contribution

ArN, SKV, and **MS** did conceptualization of the idea. The draft was written and revised by **AS, ArN, RB, PKP** and edited by **AN, RB, SK, RA, AD, PKP**, and **SKV, RB, MS, AM, RB, ANA** and **PKP** worked on illustrations. The curing of the manuscript was done by **PKP, SKV** & **MS**.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

We acknowledge infrastructure support available through the DBT-BUILDER program (BT/INF/22/SP42155/2021) at KIIT University.

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