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Animal Diseases

REVIEW

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CRISPR-Cas9 mediated phage therapy as an alternative to antibiotics

Fikre Birhanu Balcha[1] and Sultan Abda Neja[1,2*]

Abstract

Inappropriate use of antibiotics is globally creating public health hazards associated with antibiotic resistance. Bacteria often acquire antibiotic resistance by altering their genes through mutation or acquisition of plasmid-encoding resistance genes. To treat drug-resistant strains of bacteria, the recently developed CRISPR-Cas9 system might be an alternative molecular tool to conventional antibiotics. It disables antibiotic-resistance genes (plasmids) or deactivates bacterial virulence factors and sensitizes drug-resistant bacteria through site-specific cleavage of crucial domains of their genome. This molecular tool uses phages as vehicles for CRISPR-cas9 delivery into bacteria. Since phages are species-specific and natural predators of bacteria, they are capable of easily injecting their DNA to target bacteria. The CRISPR system is packaged into phagemid vectors, in such a way that the bacteria containing the antibiotic-resistance plasmid sequence or that containing specific DNA sequences were made to be targeted. Upon CRISPR delivery, Cas9 is programmed to recognize target sequences through the guide RNA thereby causing double-strand cleavage of targeted bacterial DNA or loss of drug resistance plasmid, which results in cell death. Remarkably, the safety and efficacy of this newly developed biotechnology tool and the biocontrol product need to be further refined for its usage in clinical translation.

Keywords CRISPR-Cas9, Bacteriophage, Phagemid, Antibiotic-resistant

Introduction

Globally, antibiotics have been commonly used in animals with remarkable health and economic benefits for more than 50 years (Flynn 2012). In food animals, antibiotics are used to improve growth, and to prevent and control diseases (McEwen and Fedorka-Cray 2002). In many countries, including Ethiopia, antibiotics are usually mixed with feed to promote growth in poultry farms. Since antibiotics added to feed are premixed and purchased, the amount in the feed is not thoroughly regulated (Fairoze 2012).

*Correspondence:
Sultan Abda Neja
sultanabda@gmail.com
[1] Faculty of Veterinary Medicine, Hawassa University, P.O. Box 05, Hawassa, Ethiopia
[2] Institute of Bioscience and Technology, Texas A&M University, 2121 West Holcombe Blvd, Houston, TX 77030, USA

In dairy farms, mastitis is the most common challenge resulting in economic loss and premature culling of cows. Among mastitis-causing bacteria, *Staphylococcus aureus* is measured as a major causative agent that threatens farmers, while other gram-negative bacteria that cause mastitis and milk spoilage like *Streptococcus agalactiae, Corynebacterium bovis*, *Mycoplasma* species, *Streptococcus uberis* (Erskine 2001), coliforms, *Serratia, Pseudomonas, Proteus* species, and environmental *Streptococci, Enterobacter* species are involved (Quinn et al. 2002).

In addition to affecting milk and milk products, mastitis-causing bacteria are responsible for zoonotic diseases in humans (Radostits et al. 2007). Apart from dairy health management, antibiotics are often used to control mastitis (NMC 1999). Consumption of antibiotic-contaminated milk also poses public hazards by affecting gastrointestinal microflora and the development of antibiotic-resistant zoonotic bacteria (Thirapatsakun 1999).

In general, there are many risks derived from the illogical use of antibiotics due to direct organ damage as well as the indirect influence of resistant organisms on the biotic environment (Thawani. 2010). Antibiotic resistance also leads to therapeutic failure, economic losses and animal welfare problems. Nowadays, bacteria are becoming ever cleverer by exhibiting distinct kinds of resistance even though the fight to defeat bacteria pathogens is continued (Fair and Tor 2014). As most mechanism of antibiotic resistance is through alteration of genes or acquisition of antibiotic resistance gene encoding plasmids, the issue is beyond the rational use of antibiotics and needs advanced genetic-based interventions.

In the past decade, due to its unique adaptive nature and therapeutic potential, the CRISPR-Cas (clustered regularly interspaced short palindromic repeats-Cas) system also got rising interest in the scientific community. The CRISPR-Cas9 system derived from the adaptive immune system of prokaryotes utilizes small guide RNAs (crRNAs) for sequence-specific interference with target nucleic acids. CRISPR-Cas comprises a genomic locus called CRISPR that contains short repeats separated by distinctive spacers sequences often acquired from mobile genetic elements (MGEs) such as bacteriophages, transposons or plasmids. The CRISPR array is headed by an adenine-thymine (AT-rich) principal sequence and is often separated by *Cas* genes that encodes the Cas proteins (Shmakov et al. 2015).

CRISPR-Cas systems are categorized into two classes, six types (I to VI) and various subtypes. In Class 1 systems (Type I, III, and IV) there are multi-Cas protein effector complexes while in Class 2 systems (Type II, V, and VI) there is only single effector protein (Jiang and Doudna 2017, Koonin et al. 2017). Table 1 summarizes the detail classification of CRISPR-Cas system. As bacteriophages are viruses that kill their bacterial hosts in a lytic state, they are considered as one class of therapeutics that recently attract the attention partly due to their high specificity, nontoxicity, and abundance in nature (Abatángelo et al. 2017; Abda 2020; Tang et al. 2015).

In early twentieth century, Felix ded'Herelle and Frederick Twort independently discovered the concept of 'phage therapy' and used in the treatment of bacterial infections (d'Herelle 1961). The distinctive ability of phages to specifically target living bacterial hosts was used not only for therapy but also harnessed as a tool for bacterial diagnostics, through which bacterial strains are distinguished based on their susceptibility to phages (Keen 2015). Genetic studies for constructing hybrid bacterial strains and subsequent gene mapping also utilized sections of bacterial DNA transferred by the phages through the process of transduction (Anderson et al. 1972).

Genetic modification of phage particles is carried out by genetic engineering of phage. The principle of this method is to create many phage variants demonstrating specific proteins on their surface based on the fusion of phage coated protein genes with genes encoding foreign protein or peptides fragments (Sblattero and Bradbury 2000). In the past few years, phage engineering technology has advanced the idea of phage-mediated gene therapy. Although the high specificity of phages can be useful to treat a given bacteria, Cas can increase the range bacteria to be targeted by viruses through modification made on temperate bacteriophages and phage safety while decreasing the virulent genes in the host strain. The bactericidal efficacy of bacteriophage an also be exploited by CRISPR-Cas technology to treat antibiotic-resistant bacteria (Park et al. 2017). Hence, the objective of this review is to give an overview on the mechanism of CRISPR-Cas9-mediated phage therapy, and to present CRISPR-Cas9-mediated phage therapy potential application as an alternative tool against bacterial infection.

CRISPR-Cas system

CRISPR-Cas system is a complex system first noticed by Japanese scientists in 1987 when studying genes in *Escherichia coli* (Ishino et al. 1987). Later, scientists found sequence spacers in CRISPR that are homologous to sequences in bacteriophages and plasmids,

Table 1 Features of different types of CRISPR systems

Characteristic	Type I	Type II	Type III	Type IV	Type V	Type VI
Effector complex	Multi-subunit (Class 1)	Single unit (Class 2)	Multi-subunit (Class 1)	Multi-subunit (Class 1)	Single unit (Class 2)	Single unit (Class 2)
Signature Protein	Cas3	Cas9	Cas10	Csf1	Cas12	Cas13
Target molecule	DNA	DNA	RNA/DNA	?	DNA	RNA
Details	Cleaves ssDNA strands	Originates blunt double strand break	Binds to RNA molecules	Most unknown CRISPR system	Original staggered-double strand break	RNA-guided RNase

Source: Alexandre and Gabriela. (2019)

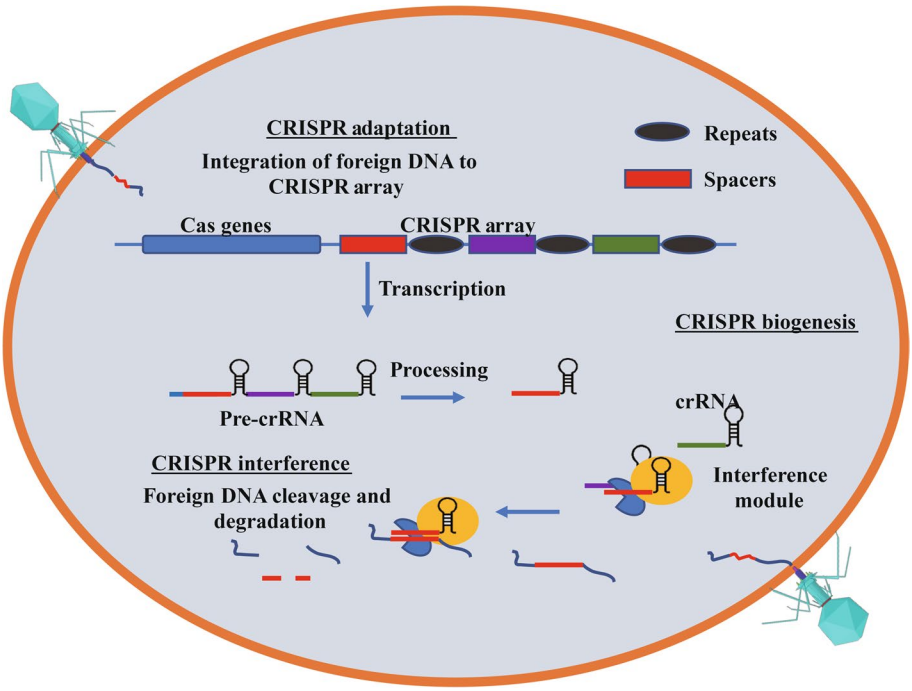

Fig. 1 The CRISPR-Cas adaptive immunity. During adaptation phase Cas1-Cas2 complex selects spacer and incorporates to the upstream of CRISPR locus. At crRNA biogenesis phase, the locus transcribes pre-crRNA which forms tracr-RNAs that helps in the recognition by RNase III and cutting process during the interference phase

indicating that CRISPR was a defense system of bacteria against such external genetic elements. The experiments in *S. thermophilus* found that strains requiring new spacers from the bacteriophage genomes allow the strains to resist the infection of corresponding phages (Grissa et al. 2007). Furthermore the bioinformatic analysis on CRISPR motifs and Cas proteins encoding sequence revealed its presence both in bacterial and Archaea genomes (Rousseau et al. 2009).

Structure of CRISPR loci

The CRISPR locus consists of short recurrence sequences of typically 28–37 bp (Barrangou and Marraffini 2014), parted by spacers each containing a distinct sequence of comparable span. The repeats are organized in a palindromic repeat but in their corresponding 5′ to 3′ directions. The spacers harboring plasmid-derived elements determine the specificity of CRISPR's defense system (Barrangou and Marraffini 2014) and also act as an immunological memory against future infection (Grissa et al. 2007).

The leader sequence often has adenine (A) and thymine (T), found upstream to CRISPR loci. This sequence has about 500 bp which includes promoter elements that signal for transcription of crRNA (CRISPR RNA) and the proper insertion of external genetic material into CRISPR sequences (Yosef et al. 2012). Next to the leader sequence there are *Cas* genes from which Cas proteins are expressed. The crRNA also transcribed from CRISPR loci along with Cas proteins forms an effector complexes that execute cleavage of foreign genetic elements.

Due to presence of variable *Cas* genes as well as diverse CRISPR loci arrangements, there are three types (Type I, II and III) CRISPR-Cas systems. However, CRISPR-Cas systems are also categorized into classes and subtypes. Class 1 with complex architecture involves multiple effector proteins to recognize and cleave foreign DNA and while class 2 system recognizes and cleaves using one multi-domain enzyme with simplified architecture. Due to its targeting specificity, adaptability and simplicity, the class 2 system that embraces the type II CRISPR-Cas9 is used in many biotechnological applications (Singh et al. 2017).

Steps of CRISPR-Cas as adaptive immunity for bacteria

Adaptation

Adaptation or acquisition is the first step for CRISPR-Cas mediated bacterial defense against invaders (Fig. 1).

Here, the genetics of the infective phage is inserted to the CRISPR-Cas, therefore giving the organism ability to recognize and further invade its phage strain. These Cas1 and Cas2 are important proteins for phage adaptation (Fig. 1) and common to all CRISPR-Cas systems, irrespective of the type (Makarova et al. 2006). Both proteins are required for this step since the expression of Cas1 or Cas2 on their own does not potentiate spacer acquisition (Yosef et al. 2012).

In the adaptation step, the Cas1-Cas2 complex plays two important roles cutting the foreign DNA found upstream of spacer (protospacer) and inserting it to the CRISPR sequence (Makarova et al. 2006). Protospacer assortment is mediated by a PAM sequence unique to each CRISPR-Cas subtype and bacteria. Additionally, PAM is also involved in the self/non-self-recognition (Anders et al., 2014). For instance, the *E. coli* type I-E CRISPR-Cas system spacer recruitment starts with the gratitude of PAM sequences in ssDNA by Cas/1a and Cas/1a' subunits (Mojica et al. 2009).

CRISPR-Cas RNA biogenesis

To sustain immunity, pre-crRNAs are produced from the CRISPR array and further processed in to crRNA (Haurwitz et al. 2010). The process is often mediated by the cas proteins or by host ribonuclease enzymes (Marraffini and Sontheimer, 2010).

Interference

The crRNA guides the CRISPR-cas machinery into foreign genetic material to be targeted and silenced. In type II system, the tracrRNA and crRNA form a complex because they contain complementary sequences (Deltcheva et al. 2011). Formation of this RNA complex leads to structural changes that activate Cas9. Once activated, the gRNA-bound complex selectively recognizes the foreign gene segment for the accurate PAM site (Fig. 1). Once recognized, the dsDNA is opened and crRNA binds to the exposed ssDNA to make R-loop to subsequently cause double-strand-break at 3 nucleotides upstream of the PAM site (Jinek et al. 2012).

Mechanism of specific DNA cleavage by CRISPR-Cas9

Among several Cas proteins, Cas9, which is a programmable RNA-guided endonuclease, commonly used for genomic editing. The crystal structure of Cas9 shows that it is a bi-lobed protein with nucleic acid recognition (REC) lobes found at the center and the NUC lobes having HNH, RuvC, and PAM-interacting nuclease domains make a grove to house the sgRNA target DNA (Nishimasu et al., 2014) (Fig. 2). Recognition of PAM needs availability of a well-matched PAM element with the

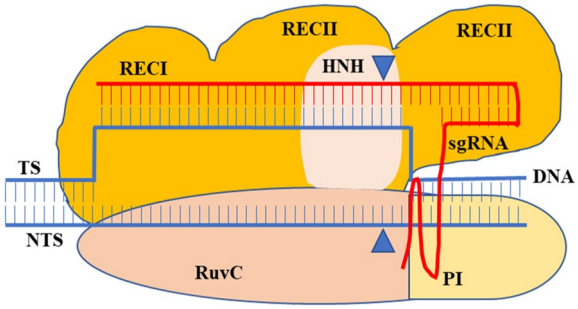

Fig. 2 sgRNA-DNA complexed with multiple domains of Cas9 protein

groove (Sternberg et al. 2014). The commencement of DNA-sgRNA hetero-duplex R-loop creation is triggered by PAM-dependent enrolment of sgRNA-Cas9 complex (Szczelkun et al. 2014).

sgRNA guides sequence complementarity formation at the target site causes allosteric activation of the nuclease domains that cause dsDNA break following the extension of the R-loops (Sternberg et al., 2015). Recent studies indicated that the conformational change of Cas9 REC lobe (REC3) during this process is important signal to the HNH nuclease to control the general catalytic ability of Cas9 (Chen et al., 2017). Cas 9 maintains an auto-inhibitory conformation as far as there is no crRNA:tracrRNA duplex. Prolonged Cas9-RNA-PAMs binding increses the Cas9 nuclease activity when it is attached to the correct target site (Anders et al., 2014).

CRISPR-Cas9 editing of bacteria

Bacterial genome editing strategy depends on the utilization of a gRNA that able to recognize a specific sequence in the chromosome where Cas9 can cut. Although bacterial genome also has some self-targeting CRISPR Cas (Stern et al. 2010), it is often deactivated by mutation. Reactivating the system is useful only if the target for cutting is pathogenicity islands or prophages from strains of interest. Counter-selecting of specific genotypes in complex populations can also carry out by Lethal self-targeting (Gomaa et al. 2013).

Nowadays, the CRISPR-Cas9 system is proposed to cut an antibiotic-resistance cassette present in another strain of bacteria. The system can also serve as another vital approach used to spare large fragments of *E. coli* genome with synthetic DNA (Wang et al. 2016). This molecular tool is already being used to explore many basic and translational studies (Liang et al. 2017, Lin et al. 2008). For instance Oh and van Pijkeren established genome-editing strategies to manipulate *Lactobacillus reuteri,* a bacteria known to have immune-modulatory and antimicrobial property (Lin et al. 2008). In general,

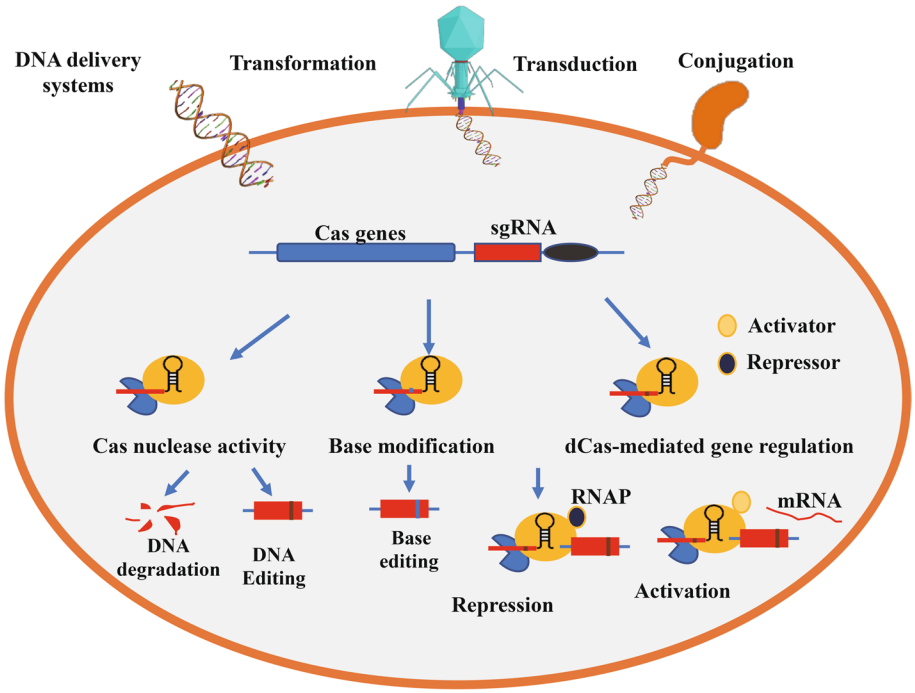

Fig. 3 Genome editing by CRISPR-Cas system. CRISPR-Cas delivery can be made *via* transformation, transduction or conjugation. The outcome can be deleterious cell death due to massive DNA degradation or, the DNA break may be repaired while introducing some mutations. Catalytic domain inactive dCas9 can also silence a target gene by interfering with RNA polymerase function

genetic modifications can be made by recombinant DNA technology that helps to construct ssNDA or dsDNA oligonucleotide that guides CRISPR-Cas9 system (Fig. 3). CRISPR- Cas9 tools have also been developed pathogen such as *Staphylococcus aureus* that often develop antibiotic resistance (Liu et al. 2017).

Anti-CRISPR mechanisms of phage

Prokaryotes harbor a extraordinary arsenal of protection plans to cohabit with their viral hunters. For instance, phages have devised diverse strategies to combat antiviral defense mechanisms. Phages can escape the CRISPR-Cas interference by making a random mutations in their spacer or the PAM sequence (Semenova et al. 2011). Furthermore, the competence of absconding CRISPR-Cas immunity by point-mutation is that spacer diversity increases the adaptive burden on the virus and therefore elicit fast death of the predator (Van Houte 2016).

New studies confirmed that Mu-like phages, that affect *P. aeruginosa*, vigorously constrain their host's CRISPR-Cas system by producing anti-CRISPR proteins that affect CRISPR-Cas interference machinery (Bondy-Denomy et al. 2015). Confiscating the host's immune mechanism is critical for phage spread as the competence of infection significantly reduces when viral spacers are deleted. Interestingly, examination of phages that able to effectively infect the host developed novel spacers that come from the similar genomic locus, consequently indicating that the virus have a entirely functional CRISPR-Cas system (Seed et al. 2013).

CRISPR-Cas9 based phage engineering

One of the main pitfalls of antibiotics is their broad spectrum of action for killing pathogens as well as normal flora bacteria (Tamma et al. 2012). Based on their genetic signatures, phages can be exploited to target and kill specific bacteria. Because CRISPR-Cas technology can be programmed so that its antimicrobials spectrum of action can be programmed and fine-tuned against specific DNA sequences of a given bacteria thereby only those bacteria that contain targeted DNA can be killed. In this way bacteria that contains genes encoding antibiotic resistance or virulence can be targeted (Bikard et al. 2014).

Phage genome editing by CRISPR-Cas system was first applied in 2014 to select a *gene1.7* mutant T7 phage (Kiro et al., 2014). Nowadays, CRISPR-Cas is widely used for phage genome editing (Fig. 4). For example, to identify *L. monocyto* genes, CRISPR-Cas based *Listeria* phages were utilized (Hupfeld et al. 2018). So, engineering the phage plasmid that has sequence area to be transcribed in to

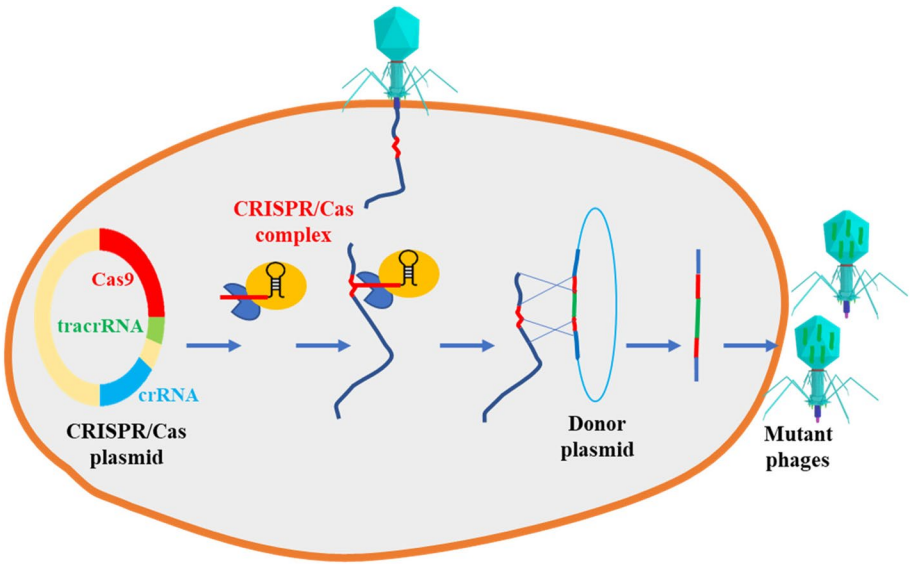

Fig. 4 CRISPR-Cas-phage engineering

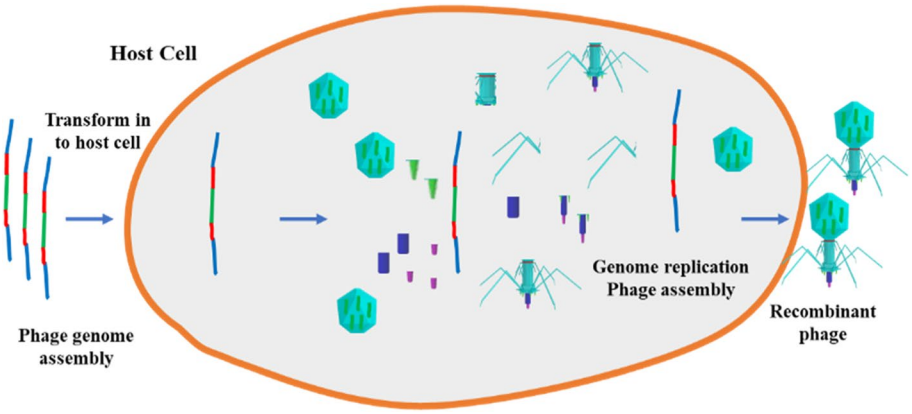

Fig. 5 Phage rebooting and assembly by genomic DNA. Host-cell machinery DNA of phage is incorporated in to the newly produced recombinant infectious phages

cas9 tracrRNA, crRNA is very important. During phage infection, the CRISPR-Cas9 complex explicitly binds and cuts the target site. Mutations are introduced into the donor plasmid and the DNA break can be repaired by recombination with the donor to generate mutants of interest (Fig. 4).

Rebooting phages using assembled phage genomic DNA

On top of the so far discussed methods which utilizes homologous recombination, an alternatively method of transforming the host cells with naked phage DNA comprising the wanted alterations, engineered phages can be directly produced (Fig. 5). Here the infectious phages are assembled in the host following production of each component. For phages of small genome like the phiX174 (5386 bp), it is possible to incorporate the genome to using in vitro produced oligonucleotides that covers full genome (Mamedov et al. 2007). For phages like T7 (39,937 bp) which has more genomes, the DNA can be assembled in vitro by ligation of discrete genome segments that were precisely cut with a given restriction enzymes (Chan et al., 2005). Through these methods phages with fragments that harbor desired mutation can be produced.

Compared to Gram-negative, the efficiency of transformation of Gram-positive bacteria is lower. Recent development however able to reboot efficiency of phages of

Gram-positive bacteria through creation of L-form bacteria so that it gets easily transformed even with bigger phage genome DNA. For instance the *Listeria* L-form has been rebooted for *Listeria* phages as well as for related bacterial genera of *Bacillus* and *Staphylococcus* phages (Kilcher et al. 2018).

Phage-mediated delivery of CRISPR-Cas9 to bacteria

To control the target infectious bacteria, efficiency of phage delivery is very important. As CRISPR-Cas system is programmable, challenge on the phage delivery can be resolved. Several studies have used phage particles as a vector to deliver DNA encoding bactericidal proteins to directly kill target bacteria without using Cas nucleases. However, such phages can also be used as DNA delivery vectors. Phagemids are thus plasmids carrying phage packaging signal, which can be repurposed to deliver different effector DNAs in to the target bacteria. Phagemids can also utilize helper phage that carries the necessary materials for the production of capsids that are not encoded by the DNA of phagemid (Dotto et al. 1981). On top of controlling the functional packaging, the modification on helper phage can also be repurposed to increase phage delivery (Russel et al. 1986).

So far, numerous toxins and restriction enzymes are delivered to *E. coli* and *S. aureus* by M13 phagemid (Bikard et al. 2014, Citorik et al. 2014, Moradpour et al. 2009). A restriction enzyme has been delivered through *Pf3* phage to control Pseudomonas (Hagens et al. 2004). The M13 phagemid system were also used to inject a plasmid encoding Cas9 as well as gRNAs that targets antibiotic-resistance genes through which efficient cell death was observed. In other study, a phagemid for Staphylococcus phage *phi1* was designed through cloning its packaging element on to another plasmid that contain CRISPR-Cas9 system. The phagemid constructed in this way confirmed to combat bacteria with various antibiotic-resistance genes and virulence factors (Yosef et al. 2015).

Using CRISPR-Cas modified phage to target antibiotic-resistant genes

Antibiotic resistance is one of the emerging public health issues across the world. There is increasing trend of prevalence of multidrug-resistant strains (Thabit et al. 2015), that demands new biomolecular tools and inventions to combat such infections. In this regard CRISPR-Cas technology has been emerged as an alternative tool to be used by incapacitating antibiotic resistance genes and bacterial virulence factors, or by producing toxic factors that kills the target bacteria (Citorik et al. 2014). For instance, researchers used Cas9 to target gene responsible for beta-lactamase resistance often found in *E. coli* plasmids.

Since the efficiency of conjugation-based technique is very poor, scientist started to use bacteriophages as a potential means to straightforwardly inject DNA into specific bacterial species (Melnikov et al. 1984). Utilizing similar approach, *E. coli* containing the antibiotic-resistance plasmid were re-sensitized to antibiotic, without any deleterious effect on wild type bacteria. The Cas9 were also made to target the gene that acts as virulence factor of Enterohemorrhagic *E. coli*. Cas9-mediated treatment were found to have better efficacy than the chloramphenicol antibiotic. Concomitantly the approach were used to combat the *Staphylococcus aureus* virulence genes (Bikard et al. 2014).

The bacteriophage-based tool to deliver CRISPR Cas9 to bacteria also against antibiotic resistance gene carried in the chromosome. This approach has been used against Kanamycin resistance gene *aph-3′* in *S. aureus* with good clinical outcomes (Bikard et al. 2014). Using mouse model of antibiotic resistant *S. aureus* skin infection were efficiently treated. Hence such approach that harness the multiplexing competences of Cas9 were able to combat antibiotic resistance factors found in the chromosomal as well as plasmid in bacteria (Citorik et al. 2014). The developed tool was also capable of target diverse sequences of a given bacteria as well as multiple bacterial species without affecting un-intended bacterial species. Such high efficiency this system over antibiotics or natural phages indicates the potential future application of CRISPR systems as alternative molecular tool to treat bacterial infection (Yu et al. 2018).

As stated above, CRISPR-Cas is an immune system of bacteria and archaea that protects them from invading nucleic acids. Bacteriophages and plasmids are inserted in CRISPR loci on the bacterial genome and later used by the Cas protein machinery to target and degrade invading nucleic acids carrying the similar sequence. Several years ago, it was postulated that a synthetic CRISPR-Cas system could be developed as an antimicrobial to kill specific bacterial genotypes (Bikard et al. 2012).

Concomitant to combating bacterial virulence and toxin production to prevent pathogenesis, antibiotic resistant bacteria can be re-sensitized to treatment. To upsurge the discerning benefit of re-sensitized bacteria, a temperate and lytic phage mediated re-sensitization were also developed (Yosef et al. 2015).

Advantage of CRISPR-Cas system in combating antibiotic resistance

DNA endonuclease Cas9 from type II system CRISPR-Cas of bacteria uses a gRNA to recognize target DNA by the base pairing principle. Such base-pairing specificity underlies the efficiency of CRISPR gRNAs dictating the Cas9 nuclease to target the sequences invader organism

(Hsu et al. 2014). This tool also allows the expression of specific anti-bacterial products that able to kills antibiotic-resistant pathogens that reside in the complex bacterial populations. Moreover, this molecular tool has selective advantage for its ability to discriminate between commensal and pathogenic bacteria.

Among the delivery methods, phage mediated delivery is the most appropriate method because of the natural infection of the phage with bacteria, for example, phage-mediated delivery during acute infection can be considered as the better methods of therapy. However, for strains of a given bacteria that has difference on phage susceptibility, an alternative phage-delivery method or vehicle is needed. If a proper and broad host vector is engineered, upgrading its delivery systems is a critical step towards tackling the challenge of the complex microbial community. There are also many other rewards that make CRISPR-phage therapy a striking alternative to antibiotics in relation to bacteriophage delivery of CRISPR-Cas9 to bacteria. For instance, unlike broad spectrum antibiotics, phages have better specificity to their bacterial hosts reducing the undesired side effects to mammalian cells (El-Shibiny and El-Sahhar 2017).

Furthermore, in terms of time and cost, the selection of new phages and the process of isolation is less expensive than the development process required for antibiotics (Golkar et al. 2014). Contrary to most antibiotics, phages have the ability to self-replicate and spread through the body during systemic administration, can pass blood-brain-barrier as well as biofilms (Wittebole et al. 2014).

Limitation of CRISPR-Cas9 mediated the phage as antibacterial biocontrol agents

The real-world environment, is not as such oversimplified and there are lots of bacteria typically embedded in a microbial community, in which using such approach often poses critics. Naturally, microbial communities residing in animal body as well as in the environment harbor diverse plasmids and MGEs bearing many resistance genes (Thomas and Nielsen 2005). If such diverse plasmids present in a single strain of species, targeting them one by one will be very challenging and needs more time and costs of downstream analyses (Marbouty et al. 2017).

Some bacteria are opportunistic pathogens some time mimicking commensal flora with desired benefits. If they are completely removed using CRISPR-Cas, the growth or metabolism of the host may be affected by its complete removal or it may allow the outgrowth of other more clinically problematic species as it happens to *Clostridium difficile* infection in the gut (Theriot et al. 2014).

CRISPR-Cas may also pose deleterious effect by increasing antibiotic resistance in some bacterial species. For instance, although the I-F CRISPR-Cas of *P. aeruginosa* successfully kills antibiotic resistant bacteria (Xu et al. 2019), the system also indorses antimicrobial resistance in *C. jejuni* (Shabbir et al. 2018). These finding showed that using this molecular tool still need advanced investigation to be used in a long-term therapeutic modality.

Another limitation of CRISPR-Cas systems is that as CRISPR-Cas9 seems easily portable between species, one of the important considerations is the reversibility of nuclease cleavage by the host repair system unless the final outcome of cleavage is host cell death. Similar to yeast cells (Tong et al. 2015), some bacteria has NHEJ systems that could repair the cleaved target DNA (Bernheim et al. 2017). Some bacteria like *E. coli* needs the exogenous recombination system (Jiang et al., 2013).

Additionally, developing and validation of Cas system against bacteria that are hard to grown in the laboratory is very difficult. The delivery vector is thus need to be redesigned so that it is able to target such bacterial species. The other limitation is that the genetic biosafety of phages is difficult to judge. Phages applied should be free from toxin or virulence factors, antibiotic-resistance genes. The function of other phage genome also needs to be analyzed for the safety before application. Also, there should be thorough mutational analysis in the CRISPR-Cas system itself once it is utilized in the host system.

Conclusion

Antibiotics are used to improve growth, and to prevent and control diseases. However, the intensive use of antibiotics for treatment, prevention, and control of disease often leads to problem of antibiotic resistance. Antibiotic resistance is often developed by alteration of host genes, plasmid, and horizontal gene transfer between bacteria. Cas9 programmed phage mediated CRISPR system degrades targeted DNA which results in cell death or plasmid loss or causes bacteria avirulent. The main problem in CRISPR-Cas9 antibacterial delivery is carrying the large protein-RNA complex across the bacteria membrane. To resolve this issue, phages are used as delivery vehicles harnessing its natural predator characteristics to the host bacteria. The efficient delivery of RGNs on conjugative vectors and phagemid to the target species bacteria and subsequent sequences-specific DNA cleavage that leads to loss of virulence, loss of antibiotic resistance and bacterial cell death are the critical characteristics of this system. Remarkably the safety and efficacy of this newly developed biotechnology tool need to be further refined to use it for clinical translation. Therefore, further research is needed to optimize the efficiency of

CRISPR-Cas and expanding the phage host range to the accurate microbial communities. The study should also be carried out by monitoring the side effects of the technology for any unpredictable ecological responses of bacterial species associated with the spread of CRISPR-Cas9 delivery vectors to the bacterial community.

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Authors' contributions
FBB: Writing and review the manuscript. SAN: Conceptualizing the manuscript, drafting the figures and editing edit the manuscript. All authors have read and approved the manuscript.

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Declarations

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