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REVIEW

Bacteriophage genome engineering for phage therapy to combat bacterial antimicrobial resistance as an alternative to antibiotics

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Abstract

Bacteriophages (phages) are viruses that mainly infect bacteria and are ubiquitously distributed in nature, especially to their host. Phage engineering involves nucleic acids manipulation of phage genome for antimicrobial activity directed against pathogens through the applications of molecular biology techniques such as synthetic biology methods, homologous recombination, CRISPY-BRED and CRISPY-BRIP recombineering, rebooting phage-based engineering, and targeted nucleases including CRISPR/Cas9, zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). Management of bacteria is widely achieved using antibiotics whose mechanism of action has been shown to target both the genetic dogma and the metabolism of pathogens. However, the overuse of antibiotics has caused the emergence of multidrug-resistant (MDR) bacteria which account for nearly 5 million deaths as of 2019 thereby posing threats to the public health sector, particularly by 2050. Lytic phages have drawn attention as a strong alternative to antibiotics owing to the promising efficacy and safety of phage therapy in various models in vivo and human studies. Therefore, harnessing phage genome engineering methods, particularly CRISPR/Cas9 to overcome the limitations such as phage narrow host range, phage resistance or any potential eukaryotic immune response for phage-based enzymes/proteins therapy may designate phage therapy as a strong alternative to antibiotics for combatting bacterial antimicrobial resistance (AMR). Here, the current trends and progress in phage genome engineering techniques and phage therapy are reviewed.

Keywords Antibiotics · Antimicrobial resistance · CRISPR/Cas9 · Multidrug-resistant bacteria · Lytic bacteriophages · Phage engineering · Global mortality · Enzybiotics

Abbreviations

AIDS	Acquired immunodeficiency syndrome
AMR	Antimicrobial resistance
CRISPY-BRED	CRISPR/Cas9-bacteriophage recombineering with electroporated DNA
CRISPY-BRIP	CRISPR/Cas9-bacteriophage recombineering with infectious particles
CRISPR-plasmid	Clustered regularly interspaced short palindromic repeats-plasmid
CRISPR-Cas	Clustered regularly interspaced short palindromic repeats-CRISPR associated proteins
CRISPR/Cas9	Clustered regularly interspaced short palindromic repeats/Cas9
DNA	Deoxyribonucleic acid
DSBs	DNA double-strand breaks
dsDNA	Double-stranded DNA
EPA	Environmental protection agency
FDA	Food and drug administration
FQs	Fluoroquinolones
HDR	Homology-directed repair
HIV	Human immunodeficiency virus
HR	Homologous recombination
MDR Bacteria	Multidrug-resistant bacteria
NHEJ	Nonhomologous end joining
ORFs	Open reading frames

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RNA	Ribonucleic acid
ssDNA	Single-stranded DNA
TALENs	Transcription activator-like effector nucleases
tRNA	Transfer RNA
UN	United Nations
USA	United States of America
ZFNs	Zinc-finger nucleases

Background

Bacteriophages (phages) are viruses that specifically infect bacterial hosts either lytically or lysogenically, or both, and are found to be the most ubiquitous microbes on Earth with single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA) or RNA genomes [1–6]. Following the discovery of powerful and efficacious antibiotics [7–12], phage therapy was lagging even though investigations on bacteriophages were not completely aborted [2, 6, 8]. Unlike antibiotics, phages offer the following advantages: (1) lower developing cost; (2) innocuous to microflora; (3) devoid of side-effects; (4) high-host specificity; (5) sustainability; (6) amenable to nucleic acids manipulation [1, 6, 13, 14]. Although phage therapy for bacterial pathogens control was established for over a century, its potentiality in mitigating bacterial infections had been greatly untapped until the rapid emergence of multidrug-resistant (MDR) bacteria [2, 8, 9, 15–22]. Bacterial antimicrobial resistance (AMR) widespread has recently led to the robust reconsideration of phage therapy for combatting AMR and biocontrol of bacterial contaminants, particularly in wastewater, food, and soil [8, 10, 11, 17, 23–26]. Therefore, the potential antibacterial properties of phages against pathogenic bacteria could be harnessed for the decontamination of biological agents in food, water, crops, wastewater, and soil, among others [1, 2, 7, 9, 17, 27–30]. By the United Nations (UN), AMR would account for 10 million deaths of persons per year by 2050 leading to economic disaster likely similar to that of the 2008–2009 global financial crisis with a high probability of dragging nearly 24 million people into extreme poverty by 2030 [17, 27, 31–34]. As of 2019, global mortality associated with AMR was estimated to be nearly 5 million which surpassed those of HIV/AIDS and malaria [17, 27, 31–34]. MDR bacteria such as *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus pneumoniae, Acinetobacter baumannii*, and *Pseudomonas aeruginosa* were recently found to be the major leading pathogens attributable to bacterial AMR [17, 27, 31–38]. AMR avails lots of opportunities for reconsideration of phage therapy as a potential tool for alleviating economic burden on public health sector not only as the 2nd -line therapy to AMR cases but also as the 1st -line therapy for biotherapeutics [17, 22, 24, 26, 35]. Due to the increasing demand for combatting AMR, regulatory restrictions on the use of antibiotics in agriculture are currently being enforced and the use of phages as strong alternatives appears to be highly rewarding for the management of AMR in the food chain [15, 17, 21, 24, 37, 39]. Studies on phages are currently being centered at understanding the selection of phage resistance and phage-host interactions to oversimplify the pharmacology of phage therapy [6, 14, 17, 30]. Although monophage therapy appeared to be more effective than multiple doses of one or more antibiotics, cocktails of phage or polyphage therapy was proven to be far more efficacious than that of monophage therapy [7, 9, 17, 27, 30, 40–43].

Recent advancements in phage genome engineering including synthetic-biology approaches, traditional homologous recombination-based phage, recombineering approaches, rebooting phage-based engineering, CRISPR-Cas (clustered regularly interspaced short palindromic repeats-CRISPR associated proteins) engineering, ZFNs (zinc-finger nucleases) and TALENs (transcription activator-like effector nucleases) appear to overcome the several limitations of phage therapy associated with natural phages as reported elsewhere [6, 14, 17, 44–47]. CRISPR/Cas9 is currently at the forefront for phage genome engineering owing to its ability to precisely overcome the limitations encountered with the wild-type phages including phage adsorption inhibition, injection blocking of phage genome, restriction-modification, abortive infection, narrow host range, among others [4, 6, 9, 14, 17, 48–52]. Harnessing phage genome engineering especially CRISPR/Cas9 for phage therapy could overcome the limitations of phage therapy exhibited with natural phages as reported elsewhere – thus reducing the economic burden on the public health sector to threat to global mortality due to bacterial AMR before 2050. Here, phage genome engineering approaches and phage therapy are highlighted and presented.

Antibiotics

Discovery and use of antibiotics

Sequel to the scientific discovery of antibiotics particularly penicillin by Alexander Fleming in 1924, the 1940 – 1970 s appeared to be the golden-era of antibiotics [7, 9, 18, 53, 54]. The discovery and development of numerous classes of antibiotics targeting the synthesis of cell-wall, folic acid metabolism, DNA replication and protein biosynthesis of the bacterial cell were successfully used to manage bacterial infections [54–56]. Unfortunately, the emergence of resistance to most antibiotics followed such that even the

powerful antibiotics – vancomycin marketed in the first quarter of the 1970s developed resistance in the late 1970s [7, 9, 17, 19, 53]. However, the emergence of resistance during the golden era of antibiotics was not challenging due to tremendous-scientific researches being conducted to overcome such resistance [17, 27, 48]. Even though, as antibiotics were widely used as therapeutics and prophylactics in many areas including healthcare, veterinary, agriculture, and industries, the rapid emergence of antibiotic-resistant bacteria is now becoming the most challenging issue particularly, to antibiotic-producing companies [17, 27, 54, 57]. The principled use of antibiotics could be thought of as follows:

- the first step involves identifying the correct clinical syndrome in patients presenting with signs and symptoms of an infection.
- identify co-morbidities that will impact etiology and antibiotic choice such as HIV, diabetes, injection drug use, and cancer.
- recognize common antibiotic resistance patterns at individual levels; carefully screen for antibiotic allergies.
- initial antibiotic therapy is often empiric and published guidelines help choose the therapy.
- antibiotic therapy should be narrowed if and when a specific etiology is determined.
- liberally use local resources to accurately manage infectious diseases and learn appropriate management [20, 54–56, 58, 59].

Although novel chemically synthesized drugs are being manufactured and marketed for combatting bacterial AMR, resistance is still emerging against almost all classes of antibiotics [7, 17, 55, 56]. In 2019, AMR caused nearly 5 million deaths associated with bacterial AMR and 1.27 million deaths attributable to bacterial AMR, which accounts for 25.66% [9, 14, 17, 24, 34]. With these drugs' limitations, phage therapy, being a biological method, can be carefully harnessed to control microbial contaminants as a strong alternative to antibiotics.

Mechanism of actions of antibiotics

The mechanism of action of antibiotics involves targeting the genetic dogma and metabolism of bacterial pathogens (Fig. 1). Antibiotics selectively interferes with various functions of bacterial cell including cell-wall synthesis, DNA replication and transcription, folic acid metabolism, or protein biosynthesis [7, 17, 20, 53–55]. The most important members of antibiotics targeting cell-wall are the β-lactam antibiotics and vancomycin [9, 53, 54, 59, 60]. Antibiotics including rifampin, chloramphenicol, macrolides, clindamycins, tetracyclines, and aminoglycosides, among

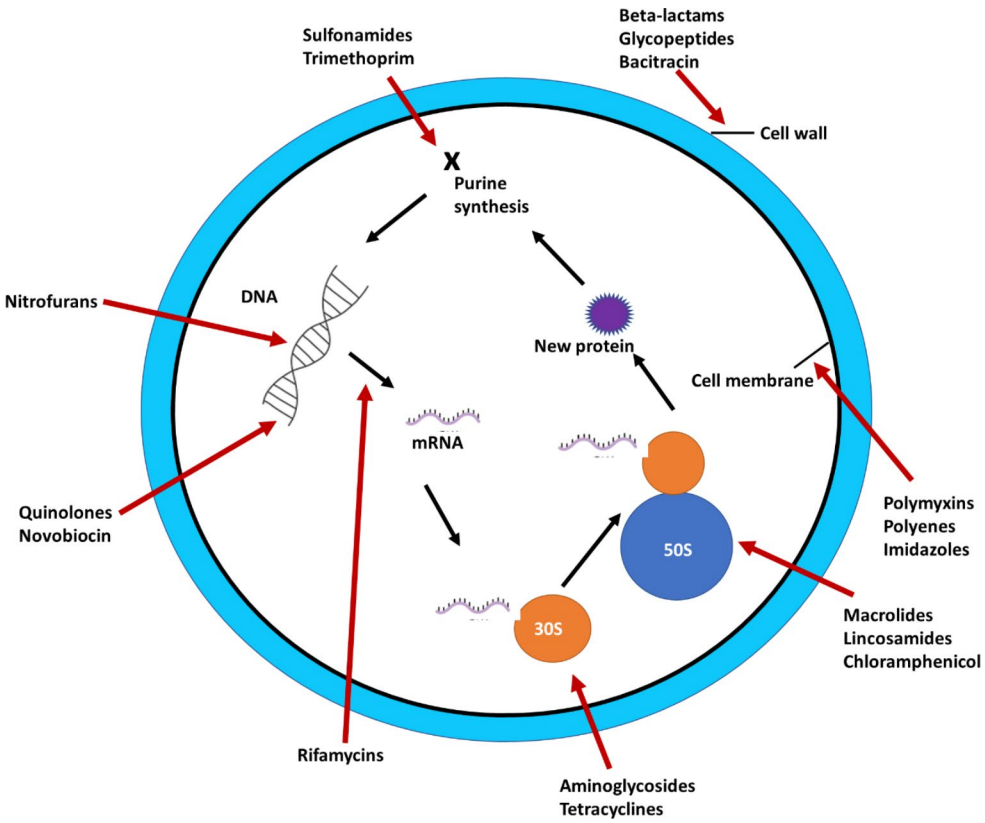

Fig. 1 Mechanism of action of antibiotics targeting the central dogma and the metabolism of a pathogenic bacterial cell

others belong to inhibitors of protein biosynthesis of bacterial cell bacteria – targeting the bacterial RNA-polymerase to a lesser extent or bacterial-ribosome to a large extent [17, 20, 53–56, 58]. Quinolones especially fluoroquinolones (FQs) are capable of antagonizing bacterial DNA replication by inhibiting the action of DNA gyrase (topoisomerase II) – thus they belong to inhibitors of bacterial DNA replication while Sulfonamides and trimethoprim are inhibitors of folic acid metabolism capable of inhibiting distinctly different steps in folic acid metabolism [55, 56]. The mechanism of action of antibiotics against a pathogenic bacterial cell is diagrammatically depicted as follows:

Multidrug-resistant (MDR) bacteria

Bacterial AMR is globally a major public health problem that is potentially deadlier than HIV/AIDS and malaria; and would account for over 10 million deaths of persons per year by 2050 if care is not taken especially in developing countries including India and Nigeria [17, 24, 31, 32, 34, 36]. A diagram of how bacteria can develop resistance to antibiotics is depicted in Fig. 2. Bacteria are capable of being resistant to a drug if and only if the optimal level of such drug that can be tolerated by the bacterial host does not end up inhibiting their growth [9, 17, 53, 56, 61]. Microbial species, especially gram-negative bacteria, are inherently capable of developing resistance to *vancomycin* [19, 56]. However, acquired resistance and selection or spontaneous mutation is used by microbial species to generate the microbial strains capable of developing resistance against a particular antibiotics which are by far reported to be responsive to such antibiotics by the microbial species in question [17, 18, 56, 61]. When these microbial strains appeared to be resistant to two or more antibiotics, they are termed MDR strains [17–20, 55, 56, 58]. The evolution of bacterial cell to antibiotic resistance, which includes antibiotic inactivation, target modification, inhibition of drug uptake, altered

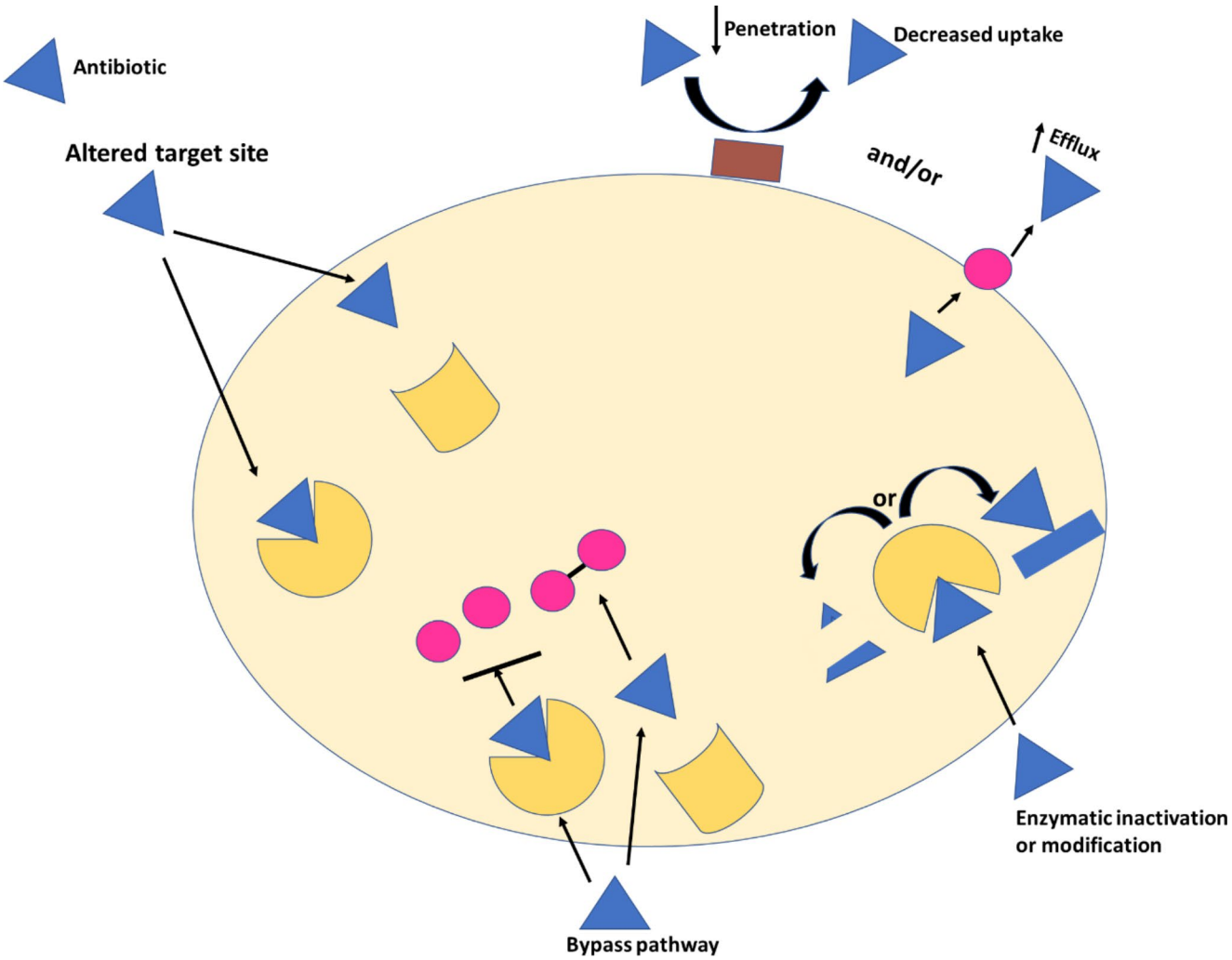

Fig. 2 Evolution of bacterial cell to antibiotic resistance

permeability, efflux, and "bypass" of the metabolic pathway can be thought of as follows:

Consequent upon above, there has been estimated a disease burden in 2019 based on global mortality attributable to bacterial AMR and associated with bacterial AMR accounting for nearly 1·3 million deaths and 5 million deaths respectively [17, 34, 36]. Therefore, investing in research and development for phage-genome-engineering technologies and phage therapy *in silico, in vitro* and in vivo will greatly reduce AMR to the barest minimum before 2050.

Bacteriophages

Discovery and use of phages

Although the discovery and use of phages against *Vibrio cholerae, Bacillus subtilis* and *Staphylococcus aureus* was established for over a century by scientists such as Ernest Hanbury Hankin (1896), Nikolay Gamaleya (1898), Frederick Twort (1915) and Felix d'Herelle (1917), the golden-era of antibiotics had led to a drastic decrease in the employment of phage therapy [2, 4, 6, 17, 40]. However, researches on bacteriophages were not completely aborted in the West due to the discovery and characterization of the viral nature of phage in 1917 and the establishment of the International Bacteriophage Institute in Tbilisi, Georgia in 1923 all together by d'Herelle [17, 27]. Lytic activities of phages were largely reported by d'He'relle; phage λ was isolated in 1951 by Esther Lederberg; the study of Smith and Huggins in 1982 revitalized researches on phages in the West leading to culture-independent approaches to phage λ in 1982 by Sanger; phage lysins' activity was demonstrated in 2001 by Fischetti; approvals of phage used in agricultural plants, food industry, and live animals were granted by United States, Environmental Protection Agency (EPA) or Food and Drug Administration (FDA) in 2005, 2006 and 2007 respectively; clearance of phage products by FDA and their phase I and II clinical trials in the USA were also granted in 2011 and 2013 respectively; in 2017, successful phage therapy for MDR bacterial infections in USA was followed [2, 8, 17, 40, 41]. The landmarks in phage history are diagrammatically depicted **(**Fig. 3**)** as follows:

Mechanism of actions of phages

Following the discovery of phages, it was known that two types of the life cycle namely: the lytic cycle and lysogenic cycle were reported [17, 27]. The mechanism of action of the lytic cycle comprises six steps namely: (1) adsorption; (2) genome entry; (3) DNA replication; (4) gene expression; (5) phage assembly; (6) host cell lysis; in contrast to the lysogenic cycle which involves (1) adsorption; (2) genome entry; (3) prophage formation [2, 6, 17, 27]. Broadly speaking, phages are similar to nanoparticles owing to their inability to replicate and divide independently. However, the ubiquity of phages in an ecosystem that supports bacterial growth initiates their adsorption to bacterial cells which

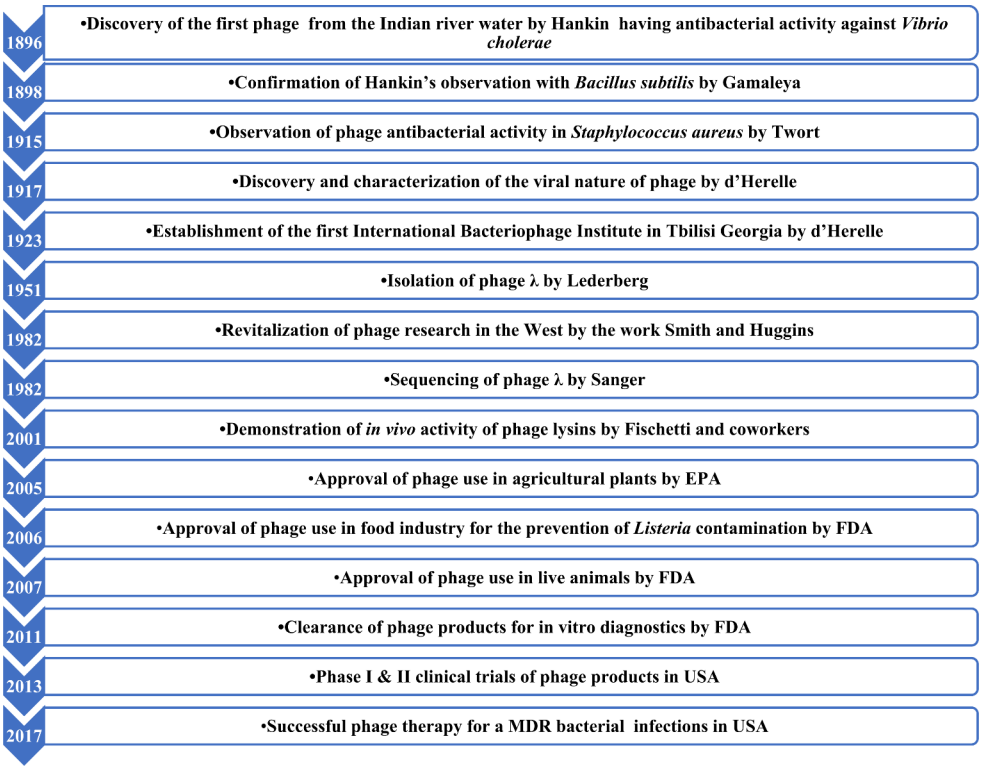

Fig. 3 Landmarks in phage history

harbour dsDNA, ssDNA or RNA of phage genome with energy, proteins and other accessory machinery to complete their lytic or lysogenic development [6, 14, 17]. Adsorption allows the phage-host interaction by random collision so that phage-host receptors which are in close proximity and right orientation interact with one another thereby injecting the phage genome into the host bacterial cell [6, 14, 17]. Phages, being the obligatory intracellular parasites of bacteria, hijack the bacterial cell machinery via either the lytic cycle or lysogenic cycle [8, 6, 14, 17]. However, the host bacteria can develop resistance against phage infection as well as at every crucial step of the lytic development by different mechanisms including phage adsorption inhibition, injection blocking, restriction-modification, abortive infection, and CRISPR-Cas systems [6, 14, 17]. Phages, equally, have evolved mechanisms for resisting countermeasures displayed by bacterial hosts [6, 14, 17]. In the lytic cycle, the phage genome does not get integrated into the bacterial host genome. Rather, it uses the cellular proteins, enzymes, and energy, among other host machinery of bacterial host cells to undergo DNA replication followed by gene expression so that the products of gene expression are assembled leading to the bacterial host cell lysis [17, 27]. In contrast, the lysogenic cycle leads to the integration of the phage genome into the bacterial host chromosome notably for horizontal gene transfer or lytic cycle depending upon the circumstance [2, 17, 27]. The mechanism of action of phages is elucidated **(**Fig. 4**)** as follows:

Methods of phage genome engineering

Biochemistry, molecular biology and genetic engineering of phages

The gigantic pieces of information culminated through culture-independent methods in the phage genome can be harnessed as a key to designing novel antimicrobial agents. Phage engineering involves nucleic acids manipulation of phages for an enhanced antimicrobial activity for pathogen control through the applications of molecular biology techniques including recombination-based techniques,

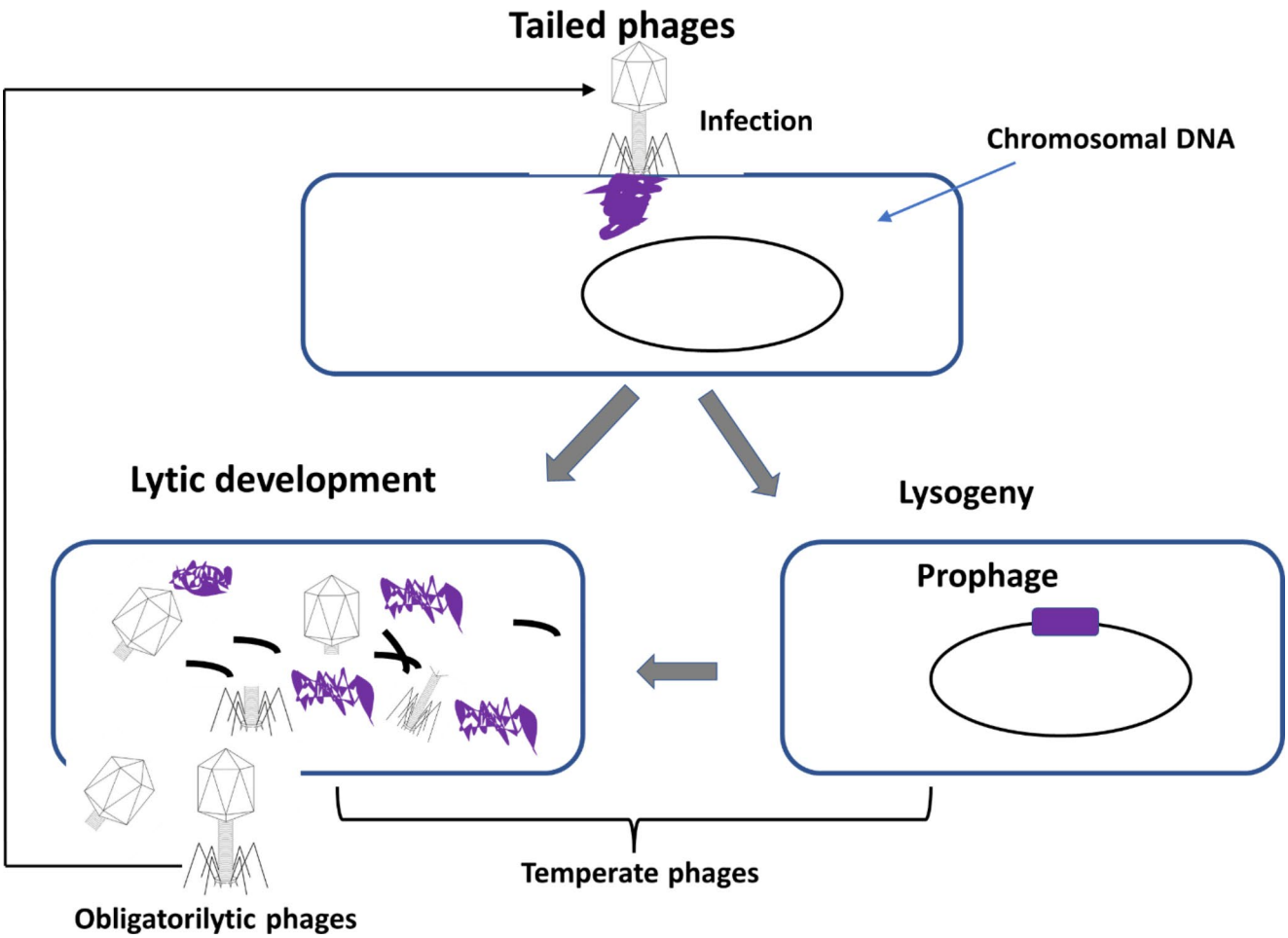

Fig. 4 Mechanism of action of phages through the lytic and the lysogenic developments

CRISPR-Cas-based engineering, rebooting phages using assembled phage genomic DNA, among others [17, 23, 43, 44, 48, 50, 52, 62, 63]. Culture-independent approaches revealed that the phage genomes could be either dsDNA, ssDNA or RNA in nature with different sizes and different ORFs (open reading frames) with or without tRNA [16, 17, 27, 50, 64–67]. Analysis of the phage genome has resulted in a dramatic understanding of phage evolution and is becoming invaluable for basic molecular biology techniques as well as for diagnosis of bacterial pathogens using luminescent phage-based, syringed-based biosensor, nanoluc-based reporter phage, among others [6, 14, 17, 57, 68–70]. The diversity of phages was estimated to be 10^{31} and phages capable of infecting and killing *Escherichia coli* species or strains appeared to be employed as models to understand the biochemistry, molecular biology, and genetic engineering of phages via electron microscopy, culture-dependent and culture-independent approaches [2, 4, 6, 14, 17, 27]. T1 – T7 phages and bacteriophage lambda appeared to be the most prevalent ones [6, 14, 17, 44, 51, 69, 71, 72]. Although phage genome engineering can be employed for antibacterial activity of wild-type phages, engineering phage genome with CRISPR/Cas9 is currently being incorporated to overcome the limitations of phage therapy such as phage narrow host range, phage resistance and any potential eukaryotic immune stimulation [6, 7, 14, 17, 41, 42, 72, 73]. Phage genome engineering strategies that involved CRISPR/Cas9 are now incorporated for phage therapy elsewhere reported to have the aforementioned limitations of phage therapy [6, 14, 42, 44]. As such, robust phage genome engineering methods designate phage therapy as a strong alternative to antibiotics for combatting bacterial AMR [17, 24, 33, 57, 69].

Synthetic biology approaches to phage genome

Synthetic biology methods of phage genome can be employed to overcome the challenges of phage resistance and phage narrow host range in vitro by manipulating the phage genome which was known to be amenable to nucleic acid manipulation with high fidelity [5, 44][17]. It involves the use of rational design, de novo synthesis and transformation tools to dramatically improve the efficiency of phage infection and to overcome the limitations associated with natural phages [44] [17]. Rationally designing and straightforward construction of phages in vitro with smaller genome size either circular or linear can produce genetically engineered phages with the potential of infecting and killing either Gram-positive or Gram-negative bacteria, or both [17, 44, 74]. Efficacy and safety of engineered phages can be tested via culture-dependent and culture-independent approaches for various applications [17, 42, 44, 49, 69, 71]. Phage genomes capable of infecting and killing the members of bacteria belonging to genera namely *Pseudomonas, Klebsiella, Bacillus, Lactococcus*, and *Mycobacterium* have been engineered using synthetic biology approaches of phage genome engineering [17, 44, 75, 76]. Owing to having orthogonal parts by phages, synthetic biology genome engineering of phages is now amenable [17, 44, 49, 74, 76]. However, the drawback of these approaches is that phages with a large, dsDNA, ssDNA, or RNA genome cannot be employed for engineering via synthetic biology [2, 4, 17, 40, 44, 49, 69].

Traditional homologous recombination/recombineering based phage engineering

While homologous recombination (HR) is traditionally a naturally-occurring type of genetic recombination by which nucleotide sequences are exchanged between molecules that share similar or identical sequences [6, 17, 44, 49, 51, 72], recombineering is a genetic engineering mediated-recombination approach developed to enhance the frequency of HR [2, 4, 17, 50]. However, the frequency of recombination appears to be unenhanced especially for Gram-positive bacteria. Infection is desperately needed for both HR and recombineering methods. In contrast to HR, recombineering requires electroporation as well as recombination proteins such as Gam, RecT, RecE, Exo, and Beta (which protect the donor dsDNA or ssDNA from bacterial hosts exonucleases) for enhancing the frequency of recombination thereby repairing DNA double-strand breaks (DSBs) in the phage genomic DNA via HR-dependent manner [17, 27, 44, 50, 63]. While the donor DNA (dsDNA or ssDNA) for HR with a single phage is incorporated in the donor plasmid, the donor dsDNA or ssDNA for recombineering approaches is not incorporated in the donor plasmid [17, 27, 44]. The donor DNA is co-electroporated with phage genome-bacterial cell after infection [27, 44, 50]. The concept of HR with two-parent phages and a single phage is depicted **(**Fig. 5**)** as follows:

CRISPY-BRED and CRISPY-BRIP recombineering approaches

Bacteriophage recombineering with electroporated DNA (BRED) and bacteriophage recombineering with infectious particles (BRIP) are recombineering techniques associated with high chances of success (4–60%) when compared with traditional HR [17, 50, 77, 78]. The dramatic improvement of BRED and BRIP recombineering technologies compared to traditional HR was known to be due to coliphage lambda proteins including Exo, Beta, and Gam as well as proteins of mycophage Che9c particularly gp60 and gp61 [38, 43, 44, 70]. CRISPR/Cas9 has been recently incorporated with

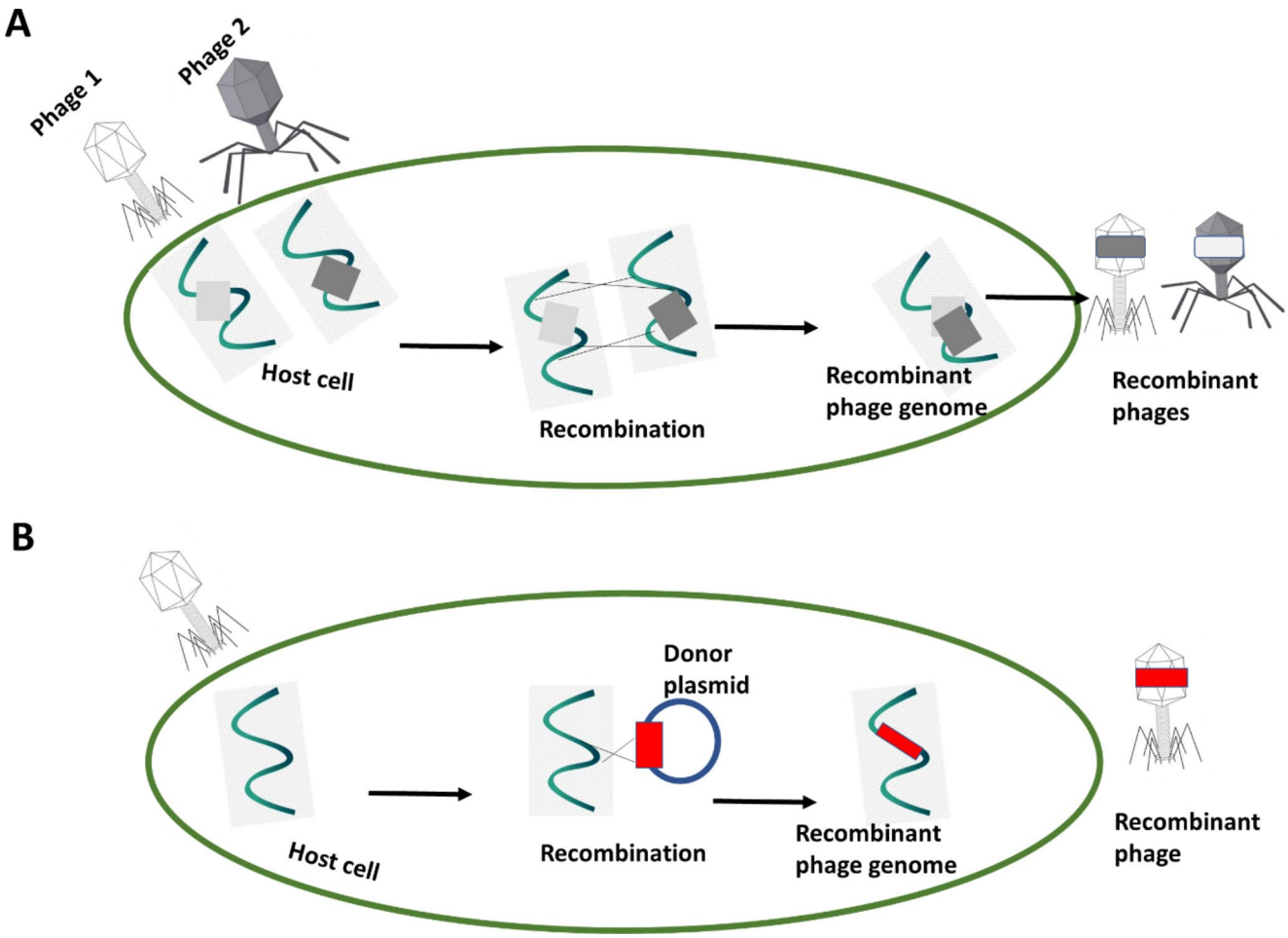

Fig. 5 Concept of HR-based phage engineering. A = HR with two-parent phages; B = HR with a single phage; white phage = wild-type phage; grey phage = wild-type phage; phages with grey & white colours = recombinant phages, phage with white & red colours = recombinant phage

BRED and BRIP technologies to enhance the efficiency and accuracy of phage genome editing via recombineering [17, 50, 78]. These powerful combinations of CRISPR/Cas9 with BRED and BRIP are termed as CRISPY-BRED and CRISPY-BRIP recombineering methods respectively. They are by far the most efficient recombineering approaches ever identified with a success rate of nearly 100% and are considered as the mutations generating systems currently developed in lytically replicating bacteriophages capable of recombineering with electroporated DNA or with infectious particles [17, 38, 50, 77, 78]. Although CRISPY-BRED appears to be more efficient than that of CRISPY-BRIP, relatively large phage genomes cannot be employed for bacterial host transfection using CRISPY-BRED technology [27, 50, 77, 78]. Thus, the need to employ CRISPY-BRIP for editing of relatively large phage genomes efficiently and precisely. Phages capable of infecting and/or killing the bacterial hosts belonging to genera particularly *Klebsiella*, *Escherichia, Salmonella*, and *Mycobacteria* are currently being employed for phage genome editing in vivo using CRISPY-BRED and CRISPY-BRIP recombineering methods [50, 78–80]. While the CRISPY-BRED recombineering approach requires co-electroporation of the phage genome and donor DNA (dsDNA or ssDNA) directly at no additional cost to infection, CRISPY-BRIP recombineering technology requires infection of a bacterial host cell with phage genome and electroporation of donor DNA similar to that in CRISPY-BRED [4, 7, 27, 50, 77, 78]. A striking feature of CRISPY-BRED and CRISPY-BRIP recombineering methods is that they are virtually similar to recombineering approaches except that infection is not required for the former and CRISPR-plasmid is not needed for the latter [27, 50]. The drawback of CRISPY-BRED and CRISPY-BRIP recombineering methods are limited to bacteria associated with extremely efficient transformation implying that host bacteria which are Gram-positive in nature do not apply to these technologies [4, 7, 17, 50, 77, 78].

Targeted nucleases for precise genome editing/engineering

CRISPR/Cas9, ZFNs, and TALENs promote genome editing by stimulating a DSB at a target genomic locus [17, 43, 44, 48, 49, 51, 52, 63]. Upon cleavage by the just mentioned targeted nucleases, the target locus typically undergoes one of two major pathways for DNA damage repair: the error-prone NHEJ (nonhomologous end joining) or the high-fidelity HDR (homology-directed repair) pathway, both of which can be used to achieve a desired editing outcome in vivo or in vitro [17, 44, 52, 63]. Of these targeted nucleases, CRISPR/Cas9 engineering appears to be the most important genome engineering tool for phages which is an adaptive immune system of bacteria gifted to fight phage genomes and other invading DNA elements on entering the bacterial cell [4, 9, 17, 38, 44, 48, 50, 51, 63]. CRISPR systems function by acquiring genetic records of invaders to facilitate robust interference upon reinfection [4, 17, 40, 44]. In CRISPR/Cas9-based phage engineering, the bacterial host cell is not only modified with the donor dsDNA or ssDNA being incorporated in the donor plasmid but also it is modified with the CRISPR-Cas9 plasmid devoted to generating the CRISPR-Cas9 complex that once formed is capable of specifically getting bound to the target site in the phage genome and thus creates a DSB during phage infection [4, 17, 44, 52, 63]. The mutations are introduced into the donor dsDNA or ssDNA being incorporated in the donor plasmid capable of repairing the DSB by recombination thereby generating mutants of interest [4, 17, 44, 51]. The concept of CRISPR/Cas9-based phage engineering **(**Fig. 6**)** is fully elucidated as follows:

Rebooting phage-based engineering

Phage rebooting entails the acquisition of activated virions from the phage genome. This approach involves assembling phage genomes with desired mutations either in vivo or in vitro followed by their transformation into the bacterial host cells through electroporation [2, 4, 17, 27, 44, 49, 50]. The replication and gene expression of genomic DNA in the host cells bring about the assembly of infectious phages [4, 17, 27, 44, 49]. Rebooting phage-based engineering is akin to recombineering-based engineering approaches except that the process is conducted without the additional cost of infection between the phage genome and bacterial host cell [2, 4, 17, 44]. The concept of rebooting phages via phage genomic DNA assembly **(**Fig. 7**)** is shown as follows:

Phage-based enzybiotics or proteins therapy

The concomitant need for the implementation of new approaches for combatting bacterial AMR has indeed justified phage therapy as a strong alternative to antibiotics. Phage therapy could now be incorporated as a powerful tool for poverty eradication due to an exponential increase in global mortality associated with AMR [17, 24, 34, 36, 38, 42, 81]. *E. coli*, *S. aureus*, *K. pneumoniae*, *S. pneumoniae*, *A. baumannii*, and *P. aeruginosa*, among others MDR bacteria were reported to be the leading pathology of global mortality attributable to bacterial AMR [6, 7, 9, 13, 14, 17, 40, 50, 55–57, 72, 81]. Phage therapy is proven to be effective particularly in tandem with antibiotic-resistant infections both in vitro and in vivo [4, 6, 9, 14, 17, 42, 57].

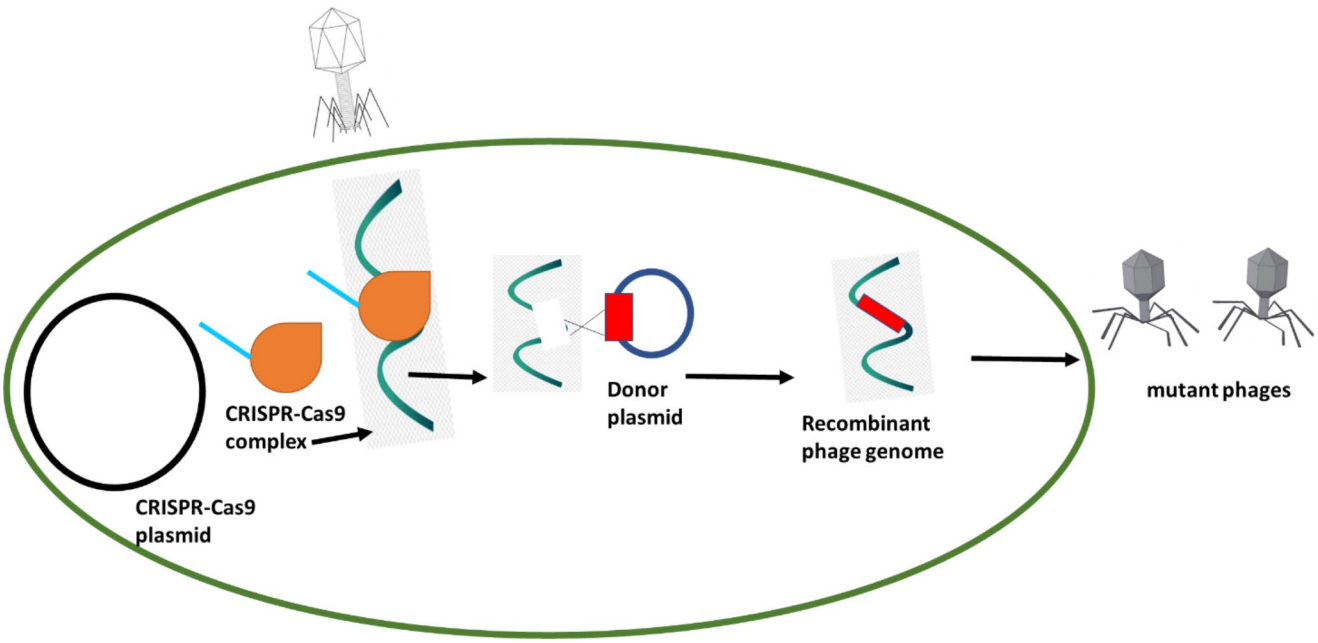

Fig. 6 Concept of CRISPR/Cas9-based phage engineering. White phage = wild-type phage; grey phages = mutant phages

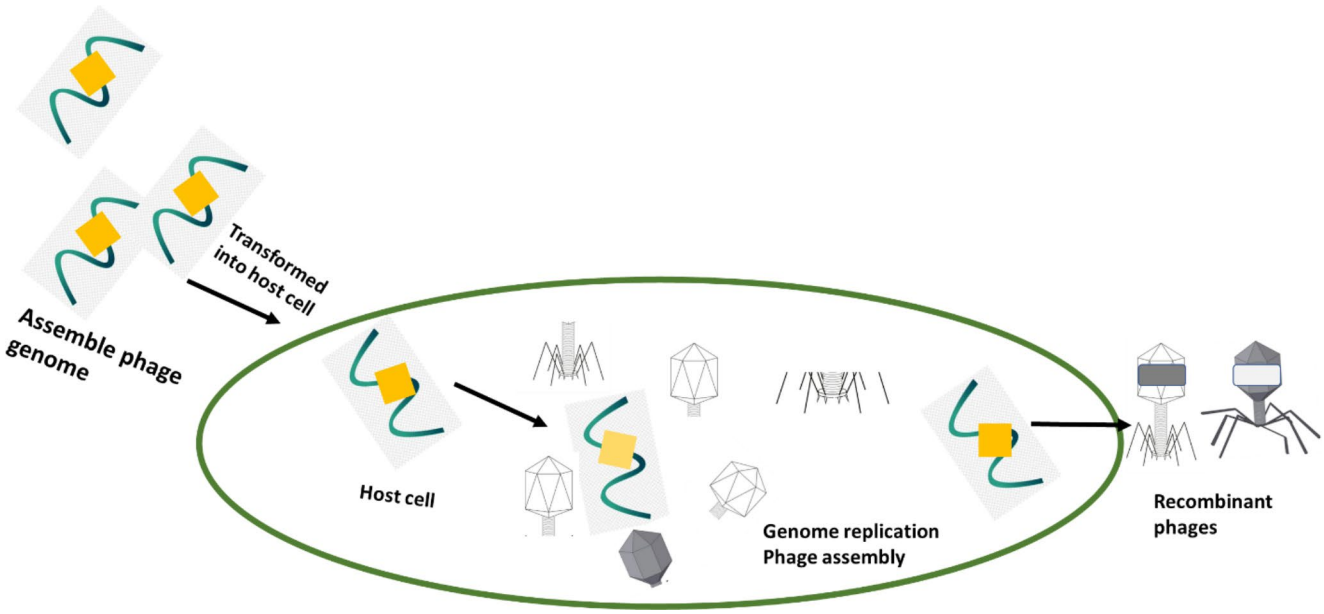

Fig. 7 Concept of rebooting phages via phage genomic DNA assembly. White phage = wild-type phage; grey phage = wild-type phage; phages with grey & white colours = recombinant phages

Phages having desirable features for phage therapy can be administered orally, locally, or by dropping a phage suspension into different parts of the body including the eye, ear, or nose to effectively treat drug-resistant disease in humans [2, 4, 17, 33, 41, 49]. These features include particularly host range, lytic activity and lack of temperate growth. Bacteria capable of producing biofilm are poorly accessible to both antibiotics and antiseptic agents, however, phages and phage-derived enzymes appeared to be the most powerful antibiofilm agents [7, 17, 72, 73]. Importantly, coliphage K29, coliphage T4, and *Listeria* phage possess enzymes capable of degrading the biofilm matrix and thus leading to lytic infection [3, 7, 17, 72, 82, 83]. In addition, phages in tandem with quaternary ammonium compounds appeared to confer a synergistic effect in degrading biofilms [2, 73]. Furthermore, phage lysins against biofilms of *S. aureus* and *S. epidermidis* were shown to be a powerful therapeutics for the control of pathogenic bacteria [2, 7, 17, 73, 84]. T7 phage via genetic engineering appeared to dramatically lower biofilm cell counts of *E. coli* by more than 99% [2, 4, 71]. This implies that phage engineering is a powerful tool in degrading biofilms by either the engineered phages or phage-encoded enzymes [2, 4, 17, 49, 72, 73]. Indeed, a lot of researches are being carried out worldwide on phages as strong alternatives to antibiotics in a wide scope of applications particularly in medicine, agriculture, food processing, and the environment for quality control monitoring of bacterial infections [9, 17, 33, 42, 50, 62, 71, 83, 85, 86]. The antibacterial proteins encoded by phages most notably peptidoglycan hydrolases and polysaccharide depolymerases are currently at the forefront for phage therapy [2, 8, 16, 17, 28, 33]. These phage-enzymes are capable of degrading bacterial peptidoglycan layer and extracellular polysaccharides including biofilm matrix, slime layers, bacterial capsules, lipopolysaccharides, among other bacterial surface polysaccharides [17, 28, 41, 83, 87, 88].

Several limitations associated with phage therapy including phage narrow host range, phage resistance and any potential eukaryotic immune response are currently being addressed following the incorporation of cocktails of phages ranging from two (2) monophages to ten (10) monophages, engineering of phage genome with CRISPR/Cas9, and encapsulation of phage enzybiotics/proteins with nano-emulsions as well as optimizing the pharmacology of phage therapy using combination therapy and selective administration [6, 14, 17, 38, 42, 57, 83, 84, 87, 89]. Therefore, a proper understanding of pharmacokinetics and pharmacodynamics with promising efficacy and safety of bacteriophage-based proteins/enzymes therapy could offer an everlasting solution to bacterial AMR.

Conclusion

Taken together, phage genome engineering especially CRISPR/Cas9 appears to be the subject of renewed molecular biology techniques towards overcoming the narrow host range of phages and the rapid emergence of resistant mutants developed by monophage therapy. Similarly, it is the most powerful and suitable molecular biology tool for developing phage cocktails or polyphages which are by far proven effective for combatting MDR bacteria. A single dose of

phage-based enzyme or protein appeared to be more effective than several doses of antibiotics, even though cocktails of phage or polyphage therapy was proven to be not only more efficacious than that of phage-based enzyme or protein monotherapy but also proved to render pathogenic bacteria blind to developing resistance against phages. Enzybiotics or proteins-derived from lytic phages for phage therapy could be used as the potential resources for poverty eradication before 2050 by saving not only over 10 million deaths of persons from AMR but also safeguarding ≥ 24 million people out of extreme poverty by 2030. Therefore, harnessing phage-based proteins/enzymes therapy and CRISPR/Cas9-based phage genome engineering for phage therapy elsewhere reported with narrow host range or phage resistance would dramatically combat global mortality due to bacterial AMR before 2050.

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