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REVIEW

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The therapeutic effect of engineered phage, derived protein and enzymes against superbug bacteria

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Abstract

Defending against antibiotic-resistant infections is similar to fighting a war with limited ammunition. As the new century unfolded, antibiotic resistance became a significant concern. In spite of the fact that phage treatment has been used as an effective means of fighting infections for more than a century, researchers have had to overcome many challenges of superbug bacteria by manipulating phages and producing engineered enzymes. New enzymes and phages with enhanced properties have a significant impact on the ability to fight antibiotic-resistant infections, which is considered a window of hope for the future. This review, therefore, illustrates not only the challenges caused by antibiotic resistance and superbug bacteria but also the engineered enzymes and phages that are being developed to solve these issues. Our study found that engineered phages, phage proteins, and enzymes can be effective in treating superbug bacteria and destroying the biofilm caused by them. Combining these engineered compounds with other antimicrobial substances can increase their effectiveness against antibiotic-resistant bacteria. Therefore, engineered phages, proteins, and enzymes can be used as a substitute for antibiotics or

in combination with antibiotics to treat patients with superbug infections in the future.

KEYWORDS
endolysin, engineered enzymes, engineered phage, multidrug resistant, superbug bacteria

1 | INTRODUCTION

Antibiotic therapy has improved remarkably in the twentieth century, however, the spread of resistance in the community is a threat to the great achievements made by available medical advancements (Friedman et al., 2016). Although antimicrobial resistance (AMR) develops naturally due to antibiotic consumption even in the appropriate and justified cases, the situation gets worse following unnecessary and excessive use of antibiotics (Laxminarayan et al., 2013). Antibiotic resistance carried by natural selection, gene transfer, and/or genetic determinants helps bacteria defend against antibiotics and thereby contributes to the survival of bacteria. In cases where antibiotic resistance is developed, a variety of broad-spectrum antibiotics may be applied, which susceptible the bacteria to multidrug resistance (MDR) via different mechanisms (Saha & Sarkar, 2021; Ventola, 2015). The acquisition of pre-existing resistance determinants and amplification in response to selection (Partridge et al., 2018), the ability of genetic exchange, for example, the transfer of a gene from the chromosome to a plasmid or between plasmids, attributed to the actions of mobile genetic elements (MGE), and processes that are involved in intercellular DNA transfer, are suggested as general mechanisms contributing to prevalent multidrug resistance. (Jeon et al., 2022). Specific segments of DNA, namely, transposons (Tn) and insertion sequences (IS), have the ability to change their location within a genome (and connected resistance genes) almost randomly to a different place in the same or another DNA molecule in a single cell (Lipszyc et al., 2022). Other DNA elements, such as integrons (In), defined as assembly platforms, cannot move and can be transported by a chromosome, plasmid, or transposon of variable length. These elements are composed of an integration site (*attI*), an integrase gene (*int*), and mobile gene cassettes (Chakravarty, 2020; Liu et al., 2006). Along with the above-mentioned features, the ability of bacterial strains to disrupt at least one of the critical stages involved in the effective action of antimicrobial agents is required to ensure bacteria survival in the presence of an antibiotic (Chinemerem Nwobodo et al., 2022). The fundamental strategies utilized by bacterial species to survive are: (i) restricting the access of antibiotics to its target site in the bacteria by decreasing its ability to enter the microbial cell, (ii) expelling antibacterial agents from the cell via the efflux pump mechanism, (iii) inactivating antibiotic through modification or degradation, and (iv) modifying or changing the antibiotic target in the bacteria (Chinemerem Nwobodo et al., 2022).

The newly evolved antibiotic-resistant bacterial species, known as superbugs, increase the duration of infection and treatment costs, which results in economic losses. Superbugs also cause hospital-acquired infections and consequently decrease the success of surgical treatments (Kumarasamy et al., 2010). MDR Gram-negative "superbugs," such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumonia*, and *Escherichia coli* are becoming a threat to the world by causing dangerous hospital-acquired and community-onset bacterial infections in humans (Jones, 2001; Sadeghi Dousari & Satarzadeh, 2021). The development of methicillin-resistant *Staphylococcus aureus* (MRSA), community-acquired MRSA, hospital-acquired MRSA, and MDR tuberculosis (MDR TB) has remarkably raised challenges for clinicians. Before the development of antibiotics, *Mycobacterium tuberculosis* was a fatal bacterial infection. Today, concern about the global epidemic following the spread of the specter of untreatable drug-resistant tuberculosis is increasing (Banin et al., 2017). The increasing resistance of important bacterial pathogens to common antimicrobial therapies and the development of MDR bacteria is reaching an alarming level (Frieri et al., 2017). Phages have special features; including no toxicity for human or animal cells, compared to conventional antibiotics, that suggest them as appropriate alternative antimicrobial agents. Their development and production also cost less than novel antibiotics (Lee et al., 2022). Phage lytic proteins are enzymes employed frequently by these phages to lyse the host. These phage lysins, which are a group of unrelated antimicrobial peptides (AMPs), are different from traditional antibiotics in their action. They act as a guided missile to kill host bacteria by causing hydrolysis of the bacterial cell wall (Fischetti, 2008). Bacteriophage-derived proteins like endolysins can be considered a potential solution to the urgent issue of antibiotic resistance. Although using phages to combat bacterial infections has been previously tried in some parts of the world, using endolysins is a relatively new idea showing great promise (Murray et al., 2021). Endolysins have been widely researched in diverse fields, including medicine, food, and agricultural applications (Wong et al., 2022). There are various types of endolysins, and their categorization is based on the structural bonds in the peptidoglycan that are cleaved by the enzyme (Borysowski et al., 2006). Different endolysin classes target the two alternative glycosidic bonds between the amino sugar moieties, namely, N acetylglucosamine and N-acetylmuramic acid (MurNAc). Lytic transglycosylases and N-acetyl-β-D-muramidases, commonly known as lysozymes, cleave the N-acetylmuramoyl-β−1, 4-N-acetylglucosamine bond, while N-acetyl-glucosaminyl-β-D-glucosaminidases hydrolyze the N-acetylglucosaminyl-β-1,4-N-acetylmuramic bond. N-acetylmuramoyl-L-alanine amidases catalyze the cleavage of the amide linkage between MurNAc and L-alanine. The importance of endolysins has increased in recent years because they exert wide lytic activities against both gram-

positive and gram-negative bacteria (Abdelrahman et al., 2021). Although phage lytic enzymes have increasingly been studied as a new treatment option for antibiotic resistance, the development of an effective phage therapy using engineered bacteriophages and endolysins has been hindered due to insufficient in-vivo studies and clinical trials. Considering the recent progress in this field, our review aims to corroborate the research and applicability of engineered endolysins and phages as an effective alternative against antibiotic-resistant pathogens and discuss challenges and limitations of natural phages.

2 | ANTIBIOTIC RESISTANCE AND SUPERBUG ERA

2.1 | Significance of MDR

There was a common interest in antibiotic therapy since its advent. Although very early therapeutic measures failed and treatments with these drugs were rejected, the continued discoveries of new classes of antibiotics eventually led to this optimism (Rima et al., 2021). According to the report of O'Neill (2016), resistant infections resulted in 700,000 deaths each year and it is expected that the number of people affected by infectious diseases will rise to 50 M globally in 2050 (O'Neill, 2016). Today, the medical community is facing greater challenges in the field of antibiotic resistance as the emergence of MDR, extensively drug-resistant (XDR), and pandrug-resistant (PDR) superbugs has made infectious diseases more difficult to treat (Moghadam, Mojtahedi, et al., 2022). MDR microorganisms acquire nonsusceptibility to at least one agent in three or more antimicrobial classes. However, XDR microorganisms are nonsusceptible to at least one agent in all but two or fewer antimicrobial classes (Alkofide et al., 2020). In recent years, the prevalence of MDR and PDR bacteria has raised significantly around the world, which has resulted in increased morbidity and mortality rates and a significant cost to society (Friedman et al., 2016).

2.2 | Superbug bacteria

The development of AMR is a complex phenomenon. The practices of health care professionals, patients' behavior toward the use of antimicrobials, and supplying chains of antimicrobials in the population may contribute to this issue (Ayukekbong et al., 2017). Infections caused by MDR and XDR superbug bacteria are of great importance because not only known mechanisms of antibiotic resistance are employed, but also these bacteria may develop resistance to new compounds following repeated or long-term administration of antibiotics (Davies & Davies, 2010). In addition, the presence of resistance genes located on the mobile genetic elements results in many different mechanisms of antibiotic resistance in the MDR and XDR superbug pathogens bacteria (Taati Moghadam et al., 2021). Further, the transmission of these genes to other bacteria leads to the spread of resistance (Taati Moghadam et al., 2021). The universal extensions of these resistant superbugs are unprecedented and likely inevitable (Deris, 2015). People with a compromised immune system such as those receiving chemotherapy are most susceptible to a "superbug" infection. Complex surgeries and chronic diseases like diabetes, asthma, and rheumatoid arthritis also make people more susceptible to antimicrobial resistant infections (Alpert, 2017). It is very hard for researchers and physicians to believe that modern-day humanity is likely to face the pre-antibiotic era and that the era of antibiotics is coming to an end (Taati Moghadam et al., 2020). Therefore, the main concern is the insufficiency of final options for treating resistant pathogens, especially superbug gram-negative bacteria that cause nosocomial and community-acquired infections (Dosari et al., 2016). A critical problem is that current clinical options are insufficient to face threats of infections caused by complex pathogens, which promotes researchers for discovering new approaches to confront the increasing problem of bacterial MDR, XDR, and PDR. Despite the implementation of different management approaches for fighting resistant pathogens, such as (a) a comprehensive understanding of the nature of resistance in terms of molecular basis, evolution, and dissemination; (b) finding new chemical agents that have antibiotic properties; and (c) enhancing the efficiency of antibiotics using innovative techniques, such as combination therapy, increasingly resistant bacteria are on the rise (Taati Moghadam et al., 2020; Yu et al., 2019). Unfortunately, the discovery of new antimicrobial agents cannot keep up with the growing occurrence of resistance. Therefore, the development of a new approach for treating infections caused by MDR and XDR pathogens is needed. However, using genetically engineered methods and the production of new antibiotics as a priority in combating infections caused by these bacteria have not been much successful (Taati Moghadam et al., 2020).

2.3 | Primary resistance

Primary resistance occurs when the bacteria face the drug of interest for the first time in a particular host (Loeffler & Stevens, 2003). Being encoded chromosomally, this form of resistance is not transmitted to other bacterial species. Although the incidence of mutated bacteria is low, in the presence of an antibiotic, susceptible bacteria are killed and mutant ones remain alive and consequently outnumber the rest of the population (Acar & Röstel, 2001; Urban-Chmiel et al., 2022).

2.4 | Secondary resistance

There is another form of resistance that only occurs in an organism after being exposed to the drug, which is called secondary resistance, or "acquired resistance" (Khalilzadeh et al., 2006; Loeffler & Stevens, 2003). Bacterial resistance may further be categorized as intrinsic resistance and extensive resistance. Intrinsic resistance, also called innate resistance, is the simplest type of resistance and occurs due to being unsusceptible naturally. This is a constant trait of a

species, strain, or whole group of bacteria. This form of resistance results from the insensitivity of a given microorganism to an antibiotic because of its "innate" resistance to specified groups of antibiotics. Lack of a receptor or low affinity for the antibiotic, impermeability of cell wall, or enzyme production are factors involved in intrinsic resistance (Irving et al., 2012). Extensively drug resistance, also known as XDR, occurs when organisms become unsusceptible to at least one or two most effective antimicrobial drugs. The treatment of patients with first-line drugs seems to result in this form of resistance, for example, XDR-TB resistance against fluoroquinolone (Lee et al., 2013; Marks et al., 2014).

2.5 | Clinical resistance

Using an inappropriate concentration of an antimicrobial agent also may result in a situation called clinical resistance. In such situations, the infecting organism is inhibited by an antimicrobial concentration that is higher than that expected to be safely achieved by normal dosing and may impair host immune function and consequently result in therapeutic failure or reappearance of infections within an organism (Tanwar et al., 2014).

2.6 | Mechanisms of superbugs

Microorganisms have developed many mechanisms to overcome the effects of drugs, and thereby stay alive in the presence of drugs. Scientific studies conducted since the mid-20th century have proposed several mechanisms that play roles in bacterial resistance to antibiotics (Tanwar et al., 2014). Bacterial antibiotic resistance results from changes in the exposure or interaction of bacteria with antibiotics. Mechanisms that remove the antibiotic actively from the cell and change the permeability of bacteria cell membranes reduce the exposure of bacteria to antibiotics and thereby result in antibiotic resistance. However, other mechanisms reduce the effects of antibiotics on bacteria by modifying antibiotic enzymes, modifying cell components that are targeted by the antibiotic, excessive expression of an enzyme that is inactivated by the antibiotic, introducing an alternative metabolic pathway, increasing the concentration of a metabolite acting as an antagonist of the antibiotic, reducing the amount or activity of an enzyme that is the activator of the precursor of the antibiotic, and even modifying regulatory systems not connected with the direct mechanism of action of the antibiotic, as well as reducing the demand for the product of the inhibited metabolic pathway (Giedraitienė et al., 2011, van Hoek et al., 2011). The emergence of new MDR strains, considered a threat to global health, seems to be related to the circulation and acquisition of already existing ARGs across different bacterial populations rather than to the emergence of new resistance genes (Jeon et al., 2022) (Bahramian et al., 2019). Mobile genetic elements (MGEs) which play a leading role in distributing AMR in many bacteria greatly facilitate the dissemination of ARGs. MGEs, including integrons, transposons, and plasmids are kinds of genetic material with the ability to shift within a genome or be transferred between strains or replicons, that allow the capture, accumulation, and dissemination of resistance genes (Partridge et al., 2018; Rijavec et al., 2006).

2.6.1 | Integrons

Integrons, as a member of MGEs, are formed from three basic elements: (i) integrase (*intI*), which is a gene encoding a site-specific recombinase; (ii) a recombination site (*attI*), recognized by the mentioned integrase, that may be associated with different gene cassettes; and (iii) a promoter (Pc), which leads transcription of genes that have been encoded by cassettes (Hall & Stokes, 1993). Integrase is involved in recruiting new gene cassettes into integrons and modifying those already inserted. Integrons can recruit a reversible cassette-associated recombination process to incorporate, and subsequently, express new genetic material in bacteria leading to new gene acquisition. This phenomenon plays a role in the evolution of bacteria by providing them with the ability to adapt to changing environments. Additionally, it is important in the acquisition and spread of antibiotic resistance, which is a matter of concern in medicine (Kaushik et al., 2018; Larsson et al., 2018; Messier & Roy, 2001). As mostly carried by plasmids or contained within a transposon, integrons as well as their mechanisms and roles played in the distribution of microorganisms have been well established and documented (Deng et al., 2015), which had also been considered to contribute to the unleashing of "Super Bugs" (Deng et al., 2015; Xu et al., 2011).

2.6.2 | Transposable

Transposable elements (TEs) are segments of DNA with the ability to change their location within a genome in an almost random pattern (Rozwadowski & Gawel, 2022). Transposons (Tns) are divided into two main groups: retrotransposons (class I) and DNA transposons (class II) (Babakhani & Oloomi, 2018). Retrotransposons are often found in eukaryotes, while DNA transposons can be found in both eukaryotes and prokaryotes (Casacuberta & Santiago, 2003). The bacterial transposons belong to the DNA transposons and the Tn family, which are usually the carrier of additional genes for antibiotic resistance (Blackwell et al., 2019). Transposons can transfer from a plasmid to other plasmids or from a DNA chromosome to a plasmid and vice versa which cause the transmission of antibiotic resistance genes in bacteria. The treatment of bacterial infectious diseases is difficult because of existing antibiotic resistance, part of which is caused by transposons (Babakhani & Oloomi, 2018). TEs (DNA Tns) are divided into four categories in bacteria: IS, composite Tns, noncomposite Tns (Tn3 family), and transposable phage Mu (Babakhani & Oloomi, 2018; Makałowski et al., 2012). Mobile elements (like DNA Tns) can cause the spread of antibiotic resistance in bacteria species (Malachowa & Deleo, 2010). Today, it has become

clear that ISs can cause bacterial antibiotic resistance in different ways (Fowler & Hanson, 2014). ISs can cause the inactivation of genes in the insertion site by direct integration, and accompanied by Tns, they cause the transmission of antibiotic resistance genes to other bacteria. For example, IS256 that exists in the composite Tns of Tn4001 is responsible for resistance to aminoglycosides. Composite and noncomposite Tns are able to increase drug resistance in bacteria by passing additional genes (such as resistance genes) (Malachowa & Deleo, 2010). Antibiotic resistance is created in most gram-negative bacteria (such as Enterobacteriaceae) by noncomposite Tns (Liebert et al., 1999). Tns are spreading across bacteria species by bacteriophages and plasmids and can diffuse drug-resistance genes (Frost et al., 2005). Recent studies have shown that DNA phages have a very significant role in transmitting drug resistance genes by horizontal gene transmission (Colomer-Lluch et al., 2011).

2.6.3 | Plasmids

Plasmids as another member of MGEs are small, extrachromosomal DNA circular molecules with the ability of independent replication (Rozwadowski & Gawel, 2022). Being most common among bacteria, plasmids can also be used as vectors in genetic engineering because they may be created artificially. Five main classes of plasmids have been defined based on their function: (i) virulence, (ii) resistance (R), (iii) fertility (F), (iv) degradative, and (v) colicin (Col) plasmids (Rozwadowski & Gawel, 2022). Colicin plasmids harbor genes for encoding proteins that are toxic to other bacteria (bacteriocins). Nevertheless, the most currently used method for plasmid classification is incompatibility (Inc) typing (Rozwandowicz et al., 2018). Most plasmids are double-stranded DNA (dsDNA), but some consist of single-stranded DNA (ssDNA) or double-stranded RNA (dsRNA) (Koraimann, 2018). Because of carrying AMR genes that can be moved from one bacterium to another, plasmids also contribute substantially to the spread of antibiotic resistance worldwide. Based on the Inc plasmid typing scheme, until now, 28 types of antibiotic resistance plasmids have been described (Rozwandowicz et al., 2018). As the most common type of plasmid in human and animal sources, IncF plasmids are principally found in *E. coli*, including UPEC (Tarlton et al., 2019). Unlike most plasmids, the IncFs are able to encode several replicons and can carry different resistance genes, including ESBL, genes that encode carbapenemases, aminoglycoside-modifying enzymes, and plasmid-mediated quinolone resistance (PMQR) genes. IncFs spread $bla_{CTX\text{-}M}$, $bla_{TEM\text{-}1}$, and bla_{NDM} genes, so are most often associated with ESBL resistance (Rozwandowicz et al., 2018; Sadeghi Dousari & Satarzadeh, 2021). The mobility of resistance plasmids is another feature used for their categorization. Those plasmids that are usually transferred between the same species are assigned as narrow host range plasmids group, the others that can easily be harbored by different bacteria are categorized as broad host range plasmids and are associated with dissemination of MDR (Mitra et al., 2021).

3 | PHAGE, ENGINEERED PHAGE, AND PHAGE ENZYMES

3.1 | The treatment properties of phage

Viruses of bacteria (bacteriophages or phages) are the most abundant biological existence on earth and produce a multitude of morphologically and genetically diverse predators that serve as natural antimicrobials (Amirheidari et al., 2022; Gordillo Altamirano & Barr, 2019; Taati Moghadam et al., 2020). The first biological source of phages was identified by Hankin in the Ganges and Jumna rivers of India and he believed that this biological agent caused changes in the cholera bacteria. Several patients with shigellosis were later exposed to this biological source, which was then introduced as the "anti-Shiga microbe" (Taati Moghadam et al., 2020). The discovery led to other researchers becoming interested in this topic and concluding the bacterial virus is responsible for bacteria destruction. It has therefore been about a century since phages have been used to fight bacterial infections (Mousavi et al., 2021). Infection machinery and receptor-binding proteins secreted by phages produce unique specificity for a given bacterial society, allowing accurate treatment of MDR infections or microbiome dysbiosis (Gordillo Altamirano & Barr, 2019; Taati Moghadam et al., 2020). It has been shown that phage treatment over 10^6/mL can change the host bacterial strain, resulting in bacterial destruction rates (Abedon et al., 2011; Lepelletier et al., 2015). The reason for attention to phages was not only the emergence of bacteria with high antibiotic resistance that limited antibiotic options to deal with the resistant bacteria but also the fact that drug companies were unable to produce new antibiotics because bacteria appeared to become resistant after antibiotic production (Mousavi et al., 2021; Taati Moghadam et al., 2020). Altogether, ideal phage for therapeutic applications pertinent to human medicine defines several aspects such as lack of cross-resistance with antibiotic classes, strong antibacterial activity to destroy the bacterial burden, sufficiently broad yet species-limited host range to attack only related bacteria, preserving the commensal microbiota, absence of inherent toxicity, and favorable interaction with the immune system with minimal immunogenicity to inhibit adverse immune responses or adjuvant capacity to enhance the immune system (Mousavi et al., 2021). It has been shown that natural phages produce effective results, but there are some limitations to their use as medicines that can be addressed by genetic manipulation (Pires et al., 2016).

First, phages usually target a limited range of strains, so mixed infections caused by multiple strains are rarely affected by phages. The use of phage cocktails can therefore solve this problem, but it is difficult to obtain regulatory approval for cocktails of diverse phages (Loc-Carrillo & Abedon, 2011). Second, phage therapy is also associated with an adverse immune response, because bacterial cells are quickly lysed and lipopolysaccharides are released (Taati Moghadam et al., 2020). Third, the lack of penetration of phages in bacterial biofilms, which are resistant forms surrounded by layers of extracellular polymer materials (Azeredo & Sutherland, 2008). The development of resistance against natural phages is possible as well.

To date, no standardized guidelines or protocols have been provided for the application or treatment of different phages (Taati Moghadam et al., 2020).

3.2 | Bacteria acquire resistance to natural phages

There are a number of mechanisms in bacteria that can resist the therapeutic power of phages. One of these mechanisms is endonucleases. These enzymes are part of restriction-modification systems (R-M), and they can go after the DNA of phages. Using clustered regularly interspaced short palindromic repeats (CRISPR) sequences, adaptive immunity is created, which destroys phage DNA (Drulis-Kawa et al., 2012; Rohde et al., 2018). DISARM or defense island system associated with restriction-modification is another antiphage mechanism widely reported in bacteria and archaea. DISARM does not let the DNA of phages enter into bacteria so it defends against phages. In DISARM, five genes are expressed, one of which has an unknown function, while four genes contribute to DNA methylation as well as the domains of DUF1998, helicase, and phospholipase D (Ofir et al., 2018). A few strains of gram-negative and gram-positive bacteria produce superinfection exclusion systems (Sie), which prevent phage DNA from entering them. In bacteria harboring lysogenic phages, these systems bind to membranes or elements of the membrane, preventing subsequent infections by other phages (Labrie et al., 2010; Susskind et al., 1971). Innate immunity of bacteria, called abortion immune systems (Abi), inhibits the spread of phages by killing bacterial cells prematurely after phage infection by blocking phage multiplication. Abi systems include many bacterial toxin-antitoxin systems encoded on chromosomes and plasmids. As a result, "altruistic suicide" protects uninfected surrounding cells by killing infected cells and keeping the phage population alive (Chopin et al., 2005; Fineran et al., 2009; Samson et al., 2013). However, genetic mutations can destroy receptors necessary for phage absorption in bacteria, so those bacteria cannot be infected by phages. A physical barrier, such as extracellular polymers and capsules, is often found to shelter phage receptors, as well. Thus, physical barriers can not only prevent bacteria from killing themselves under adverse conditions, but they may also prevent phages from finding receptors (Taati Moghadam et al., 2020). A method by which bacteria methylate their DNA, preventing phages from replicating, is known as bacterial phage exclusion (BREX). The BREX has six genes that, if horizontally transferred, can provide extensive phage resistance against a wide range of lytic and temperate phages (Bhushan, 2018). In a limited population of bacteria, phage receptors are produced slowly and at low levels, resulting in long-term stability, as well as phage-resistant mutants (Chapman-Mcquiston & Wu, 2008). Another antiviral system where RNases are more effective in starvation conditions is the production of RNase III and MazF in the Bacillus species. Incomplete infection can result from RNase secretion when it interferes with phage absorption on one hand and causes phages not to be absorbed on the other (Mahmud et al., 2016). A phage-inducible chromosomal island (PICI) is a genetic element in bacteria with high mobility that interferes with phage reproduction. In spite of the fact that their mechanism of action is still unknown, PICIs are remarkably implicated in host adaptation, horizontal gene transfer, phage parasites, and virulence (Martínez-Rubio et al., 2017; Penadés & Christie, 2015). Quorum-sensing regulation (QSR) is a mechanism that allows bacteria to cope with infection of different phases depending on the density of their population. It has been shown that QSR downregulates phage receptors when bacteria are at high cell density, resulting in resistance to phage. However, when bacteria were at low density of cells, QSR did not affect receptor expression and bacteria were completely susceptible to phages (Hoque et al., 2016; Tan et al., 2015).

3.3 | Engineered phage

Through genetic modification, synthetic phages can overcome most of the limitations mentioned above. Biotechnological and genetic engineering of natural phages involve integrating foreign genes, replacing genes, and mutating genes using molecular techniques to expand the host range or increase antibacterial activity. Mutations and gene replacements generally occur in the genes associated with the tail fiber protein, which is responsible for extending the host range of phages. The products of foreign gene integration are usually detrimental to the phage, even though the genes are integrated on nonfunctional phage regions (Guo et al., 2021). Genetically manipulated phages were obtained from variety of technique—homologous recombination including whole-genome synthesis and assembly from synthetic oligonucleotides, clustered regularly interspaced short palindromic repeats (CRISPR)-Cas-mediated genome engineering, traditional homologous recombination-based techniques, yeast-based assembly of phage genomes, bacteriophage recombination of electroporated DNA, in vivo recombination, cell-free transcription-translation systems, and in vitro manipulation of phage genomes (Chen et al., 2019; Pires et al., 2016). Specifically, engineered phage therapy differs from conventional therapies in that it involves seven main pillars (Figure 1). As an example, the host range modulation mechanism allows phages to expand or adapt their host range when receptor-binding proteins are exchanged between them. (Dunne et al., 2019). As a strategy for enhancing antibacterial efficacy, antimicrobial encoding genes can be combined into therapeutic "payloads" to develop secondary antibacterial agents and facilitate the killing of phages (Krom et al., 2015; Lu & Collins, 2007). To produce nonlytic phage variants that kill their bacterial hosts with less endotoxin production to avoid the induction of adverse inflammatory responses, holin-endolysin system genes can be deleted through immunomodulation mechanisms to produce nonlytic phage variants (Matsuda et al., 2005; Meile et al., 2022; Paul et al., 2011). Moreover, phages can be reprogrammed to release alternative immune responses at the site of infection or used as gene therapy, drug delivery, or vaccine adjuvants (Matsuda et al., 2005; Meile et al., 2022; Paul et al., 2011). In a companion diagnosis, phages are used as a

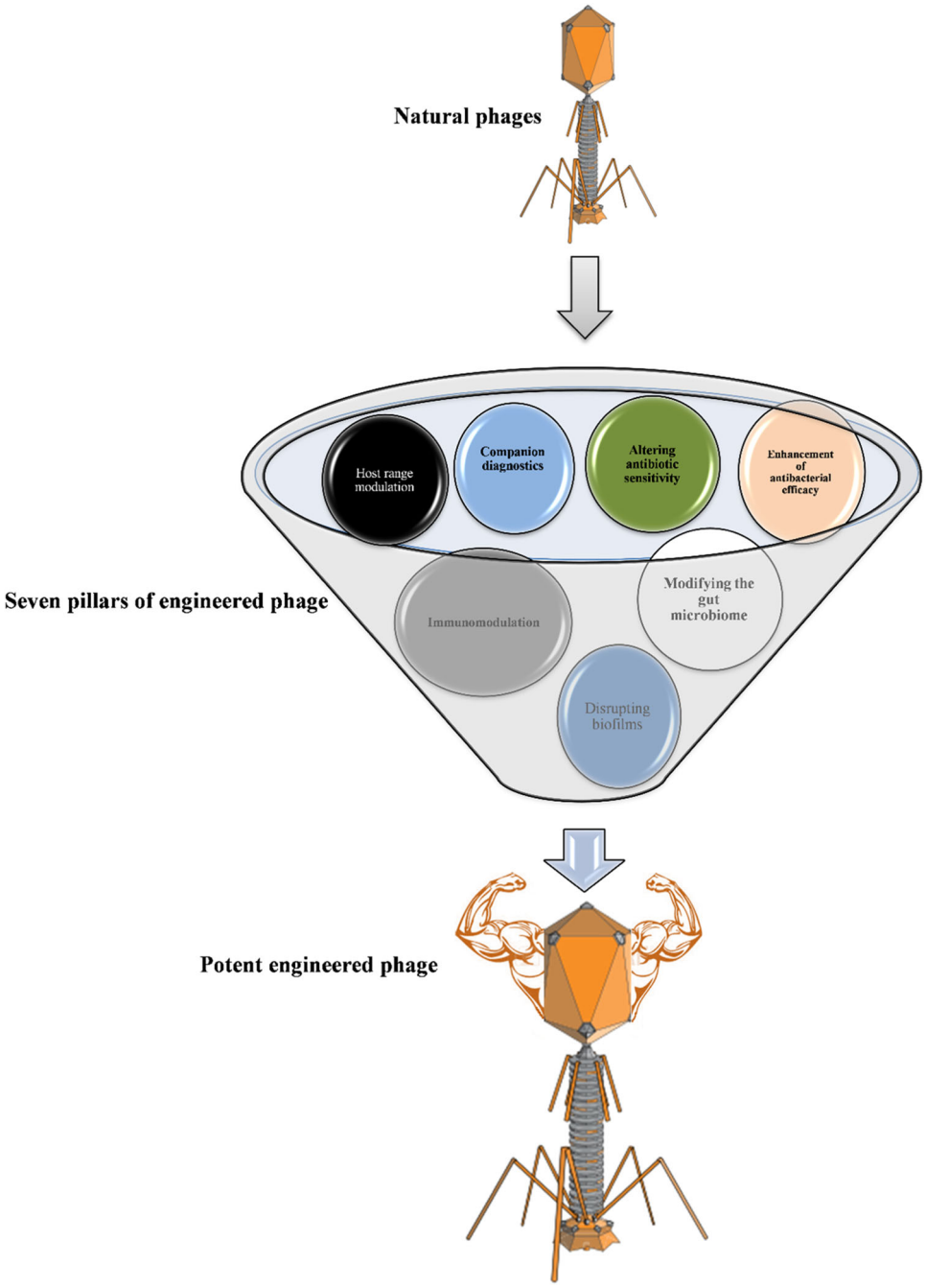

FIGURE 1 By passing a natural viral pathogen through the genetic engineering filter, the phage undergoes changes in one or more of the seven main goals of genetic engineering, resulting in a powerful phage that is useful, especially for eliminating superbugs.

diagnostics agent. For example, luciferases can be integrated into phage genomes to allow sensitive, direct, and rapid detection of target bacteria (Meile et al., 2020). A fascinating field is the modification of the gut microbiome, since it is well understood how disturbed communities of the body's microbiota negatively affect human health. Therefore, it is vital to provide solutions for adjusting the composition of the microbiota. Phages are an appropriate solution for balancing the microbiota community. Interspecies signals balance the growth of bacteria in the body's microbiome, positively or negatively depending on how they interact with other members of the microbiome. By reducing the number of species targeted by bacteriophage, the population of nontarget species is balanced, but there is an inherent risk that these species will become resistant to phages. A genetically modified phage, however, was an appropriate strategy for balancing the composition of the microbiota communities (Gibb et al., 2021; Hsu et al., 2019; Moghadam, Bakhshayesh, et al., 2022; Taati Moghadam et al., 2022). By disrupting the biofilm mechanism, bacteria can become multicellular, attached to various surfaces, including living tissues, medical devices, food, water pipes, and industrial equipment by an extracellular matrix polysaccharide.

In biofilms, polysaccharides form a physical barrier that makes them resistant to antimicrobial agents (Lu & Collins, 2007; Pei & Lamas-Samanamud, 2014). Capsule-producing bacteria are susceptible to phage infection, and capsules contain the same ingredients secreted by depolymerase enzymes (an enzyme that breaks down polysaccharides). Generating depolymerase enzymes from phages that lack this ability can be accomplished through genetic engineering. Engineered depolymerases are an effective strategy to improve phage efficiency on bacterial biofilms since they are specific for the type of polysaccharides to be degraded (Lu & Collins, 2007; Pei & Lamas-Samanamud, 2014). This highlights the fact that different phage components can be engineered to carry payloads to enhance antibiotic sensitivity. It was shown that repressing the SOS DNA repair system in the phage improved the bactericidal activity of ofloxacin when it induces DNA damage by, for example, modifying the genes in the phage. Moreover, the engineered phage was even effective at degrading antibiotic-resistant bacteria (Lu & Collins, 2009).

3.4 | Engineered phage enzymes and derived protein

It has been shown that phage lytic enzymes can substantially reduce the incidence of infectious diseases when used against a wide range of hosts because they are effective against a wide variety of pathogens (Oliveira et al., 2012; Rodríguez-Rubio, Gutierrez, et al., 2016). They can be divided into two categories, endolysins and virion-associated lysins, which are part of a broad class of enzymes called "enzybiotics." An endolysin is a lytic enzyme that attacks peptidoglycans and destroys bacterial cells (Maciejewska et al., 2018). Virus progeny is permitted to spread by an enzyme expressed late in the replication cycle of the virus. Virion-associated lysins, on the other hand, bind to virion particles and destroy the cell surface from outside, allowing the phage to inject its genetic material into the infected bacteria (Maciejewska et al., 2018; Nelson et al., 2001). A lysin associated with a virion may also be called an exolysin, a tail-associated muralytic enzyme, a tail-associated peptidoglycan hydrolase, a tail-associated lysin, or a structural lysin (São-José, 2018). At first, there was a mistake thinking that enzybiotics relied mainly on endolysins attacking gram-positive pathogens because gram-negative bacteria have an outer membrane that limits the availability of lytic enzymes to the murein layer of peptidoglycan, but after a while they were found to be effective on gram-negative bacteria as well (São-José, 2018). In contrast to endolysins, virion-associated lysins are emerging as promising enzybiotics revealing several potential benefits. Aside from clinical applications, phage lytic enzymes can also be used to control pathogens in the food industry, diagnostics, and agriculture (Oliveira et al., 2012; Rodríguez-Rubio, Gutierrez, et al., 2016). Two decades ago, the effectiveness of endolysin was examined in vivo in animal models of bacterial infection. Most of the early studies on local application of this enzyme for decolonization and general systemic infection relied on mice infected intraperitoneally. In recent years, phage lytic enzymes have been evaluated as a systemic treatment for more specific and complex infections, such as deeper tissue penetration, abscesses, biofilms, and intracellular bacteria (Schmelcher & Loessner, 2021). A wide variety of infections can be treated with phage lytic enzymes, including oral, intratracheal, intramammary, intramuscular, intravenous, intranasal, topical, subcutaneous, and intraperitoneal infections (Schmelcher & Loessner, 2021).

Although engineered phages and enzymes have some disadvantages, such as being applicable mainly to phages of small genomes whose replicative form is circular dsDNA, requiring highly competent host cells for transfection with large phage genomes, occurring only with plasmid-cloned DNA fragments as donors for recombination, requiring an R-M-deficient and transformation proficient bacterial host, modification of various phage genome regions with donor DNA each time requiring cloning of relevant spacer in the CRISPRCas source plasmid, low recombinant recovery of obligatorily lytic phages, requiring complex methodology and difficulty, and obtaining genetically engineered phages being impossible if phage genomes recovered from yeast cells are nonfunctional, their numerous benefits make them an interesting option for treatment (Łobocka et al., 2021). The modification of phage lytic enzymes has improved their effects (Figure 2), making them more effective at penetrating the outer membrane of gram-negative bacteria. An effective strategy to reach peptidoglycan is to combine a phage lytic enzyme with a compound that attaches to the outer membrane (De Miguel et al., 2020). Pesticin is a lytic toxin produced by fusing phage T4 lysozyme with pesticin. Specifically, pesticin binds to a transporter in the outer membrane of cells, resulting in phage T4 lysozyme being effective in killing cells expressing that transporter (Lukacik et al., 2012). The Artilysin® enzyme is another engineered phage lytic enzyme that has emerged to

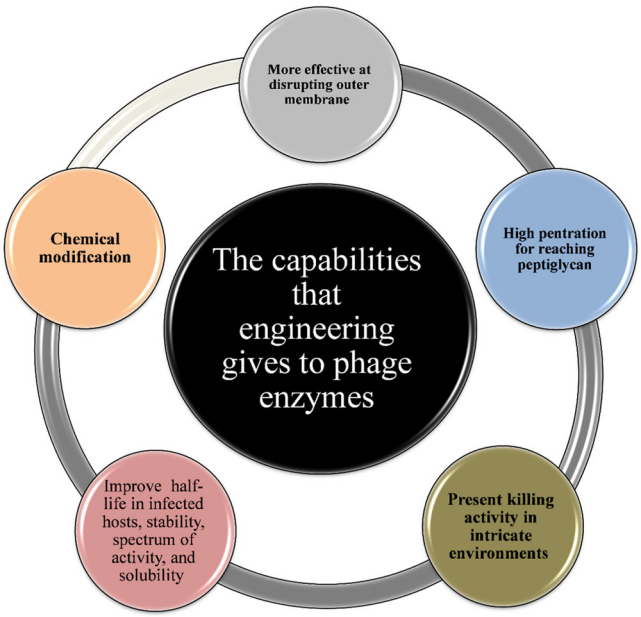

FIGURE 2 An important advantage of genetic manipulation is that enzymes isolated from phages are capable of acting in a targeted manner and with increased capacity.

overcome the limitations of natural phages against bacterial infections. The Artilysin® is the product of the fusion of another endolysin with a peptide capable of destabilizing LPS, which can penetrate the peptidoglycan and perform their function there. There is no impact on the secondary or tertiary structure of the enzyme from this peptide, which binds either to the C- or to the N-terminus of the enzyme (Gerstmans et al., 2016). There is another generation of engineered phage lytic enzymes, namely phage chemical modification that has emerged when abundant novel chemical substances have been created. These substances such as silver nanoparticles, AIEgens, pheophorbide A, cellulose membrane, and indium tin oxide are completely novel for bacteria and may affect bacteria in a variety of ways, so bacteria do not have adequate resistance genes to cope with these substances (Guo et al., 2021). Recombinant phage lytic enzymes in a medicinal landscape will usually need to have killing activity in intricate environments which include mucosal membranes, animal tissue, body fluids, and blood. Aside from optimizing killing performance, phage lytic enzymes can also be manipulated to improve other properties, such as stability, spectrum of activity, solubility, and half-life in infected hosts (Gerstmans et al., 2018). Although due to its proteinaceous nature and capacity to induce lysis of phage lytic enzymes on bacteria is expected to develop host immune response along with the generation of antibodies that neutralize this therapeutic enzyme, it was interestingly determined that antiphage lytic enzyme antibodies did not have any significant adverse effects and had a low antimicrobial activity effect (Gerstmans et al., 2018). It has become a relatively conventional treatment option for bacterial infections resistant to a wide range of antibiotics with the use of custom designed and engineered phages and phage enzymes.

4 | ENGINEERED PHAGE, DERIVED PROTEIN AND ENZYMES AGAINST SUPERBUG BACTERIA

In the clinic, superbug bacteria have led researchers to discover new substances with antimicrobial properties due to the challenges they pose. In this way, researchers have taken effective steps by using engineered phages and their enzymes as one of the best solutions. In several studies, engineered phages and enzymes have been used against bacteria that are not even antibiotic resistant, including *Helicobacter pylori*, *S. aureus*, *E. coli*, and *streptococci*. However, the main objective of engineering phages and enzymes is to make them more effective against superbugs that are resistant to antibiotics (Cobb et al., 2019; Rodríguez-Rubio, Chang, et al., 2016; Westwater et al., 2003; Xu et al., 2021).

4.1 | The effect of intact and engineered phages against superbugs

Emslander et al. (Emslander et al., 2022) demonstrated a general cell-free platform for advancing production, proteomic characterization, and transient engineering of phages in 2022. One-pot reactions containing a few microliters produced impressive phage doses against *K. pneumoniae*, *Y. pestis*, and enteroaggregative *E. coli*. The nongenomic phage engineering procedure has therapeutic activity against MDR bacteria because it adds functionality for only one replication cycle. As part of another study, an engineered three-phage cocktail was administered to a 15-year-old patient with cystic fibrosis who had a disseminated drug-resistant *Mycobacterium abscessus* infection. A lytic phage intravenous treatment successfully killed an infectious *M. abscessus*, was well tolerated, and was associated with objective clinical improvement, including improved liver function, wound closure, and substantial improvement in skin nodules infected with infection (Dedrick et al., 2019). In a recent research study created EATPs as an engineered anti-CRISPR (*Acr*) gene-containing phages by introducing *AcrIF1*, *AcrIF2*, and *AcrIF3* genes as Type I anti-CRISPR into the phage DMS3/DMS3m to present the ability for inhibiting *P. aeruginosa* infection and replication. In this way, impressive antibacterial properties as well as high safety were achieved when fighting MDR *P. aeruginosa* isolates. The EATPs also significantly reduced antibiotic resistance associated with infection by highly antibiotic-resistant bacteria (Qin et al., 2022). AIEgens-PAP is a novel form of antimicrobial biological conjugate (AIEgens-PAP) constructed by He et al. (He et al., 2020) by using the photosensitizer AIEgens with photodynamic inactivation function. Recombinant AIEgens bound precisely to *P. aeruginosa* and produced ROS, as well as exhibiting an enhanced bactericidal activity. Additionally, the AIEgens–PAP-modified phage was highly effective at curing skin wounds caused by MDR *P. aeruginosa*.

4.2 | Phage related proteins and enzymes against superbugs

Art-175 was used by Defraine et al. (Defraine et al., 2016) in 2016 to treat MDR *A. baumannii* isolates. A modified form of endolysin KZ144 with an N-terminal SMAP-29 fusion was included in Artilysin Art-175. According to the results, Art-175 was highly effective in killing MDR *A. baumannii* isolates, even those resistant to last-line antibiotics like colistin. Using colistin-resistant mcr-1-positive coli isolates, Schirmeier et al. (Schirmeier et al., 2018) studied the bactericidal and inhibitory effects of endolysin encoded by bacteriophages as Artilysin Art-175. The Art-175 antimicrobial function was high in colistin-resistant *E. coli* with *mcr*-1 gene. Based on reports, Art-175 does not show cross-resistance with colistin, and that the number of colistin-resistant bacteria containing *mcr*-1 gene was reduced. A study conducted by Thummeepak et al. (Thummeepak et al., 2016) examined the lytic activity of LysABP-01 endolysin from bacteriophage ØABP-01 against MDR and XDR *P. aeruginosa*, *A. baumannii*, and *E. coli* strains. In addition to degrading bacterial cell walls, LysABP-01 works synergistically with various antibiotics, including colistin, against drug-resistant strains of *A. baumannii*. In a study, Ply6A3 endolysin from the vB_AbaP_PD-6A3 phage was tested for its ability to deal with MDR *A. baumannii*. Ply6A3 endolysin disrupted not only MDR *A. baumannii* isolates, but also other strains containing coli, methicillin-resistant *S. aureus*, and lethal sepsis of

mice via *A. baumannii* was effectively rescued by intraperitoneal injection of Ply6A3 endolysin (Wu et al., 2019). Using phage cocktail and endolysin, Jasim et al. (Jasim et al., 2018) assessed the therapeutic efficacy of phage cocktail and endolysin against the MDR, XDR, and PDR *A. baumannii*. A decline of bacterial density (>1 log) in just 1 h of endolysin treatment was shown, and most XDR and PDR isolates were efficiently degraded by endolysin. A study was conducted in 2015 to analyze the antimycobacterial properties of endolysins. Endolysins derived from BTCU-1 mycobacteriophages were used. BTCU-1 contains two lytic genes, *lysA* and *lysB*, which have been identified, subcloned, and expressed in *E. coli*. In both LysA and LysB, Mycobacterial cell shapes were significantly modified to inhibit smegmatis growth. Further, LysA or LysB significantly reduced the number of viable intracellular bacteria in bactericidal activity assays (Lai et al., 2015).

Deng et al. (Deng et al., 2021) studied the killing activity of LysO78 endolysin against antibiotic-resistant bacteria. Various genera and species of bacteria, including *Klebsiella*, *Pseudomonas*, *Salmonella*, *Burkholderia*, *Shigella*, *Yersinia*, *A. baumannii*, *Chitinimonas arctica*, *Ralstonia solanacearum*, and *E. coli*, were shown to exhibit broad-spectrum bacteriolytic activity by LysO78. Furthermore, the LysO78 peptidoglycan hydrolases showed particularly high activity against MDR *E. coli* APEC O78, so it is considered a promising therapeutic agent for nosocomial infections and MDR *E. coli*. The RL_Lys endolysin and RL_Hlys (holin fused at the N terminus of endolysin) from the RL phage of *P. aeruginosa* affected on a wide range of bacteria including; MDR *P. aeruginosa*, *K. pneumoniae*, methicillin-resistant *S. aureus*, and *Salmonella*. As a result of the holin in the N terminus of RL_Hlys, it was more effective than RL_Lys because it enabled endolysin to reach the bacterial cell wall (Basit et al., 2021). As outer membrane-penetrating endolysins, (Briers, Walmagh, Van Puyenbroeck, et al., 2014) developed and optimized an approach to deal with Gram negative bacteria such as *P. aeruginosa* and *A. baumannii* using engineered Artilysins. MDR isolates were killed in vitro with a 4–5 log reduction by artilysins combining a modular endolysin with a nonapeptide polycationic. An increasing linker between the peptide and endolysin can further enhance the ability of artilysins to degrade peptidoglycan after passing through the outer membrane. An endolysin from phage LPSE1 has been tested as a means of combating MDR *Salmonella* strains in a research study. Researchers found that 0.1 m recombinant LysSE24 (His-tagged LysSE24) for up to 5 min significantly modified the cell shape of *Salmonella* and had antibacterial activity against 23 tested MDR *Salmonella* strains (Ding et al., 2020). By combining endolysin and virion-associated peptidoglycan hydrolases with the SPK1 signal peptide, Chandran et al. (Chandran et al., 2022) produce recombinant lysine proteins. MDR *S. aureus* was lysed by Endo88 (recombinant endolysin) and VAH88 (recombinant virion-associated peptidoglycan hydrolases), but Endo88 was more effective. In a study by Plotka et al. (Plotka et al., 2019), Ts2631 endolysin (from the extremophilic Thermus scotoductus) was tested against MDR *P. aeruginosa*, *A. baumannii*, and pathogens from the *Enterobacteriaceae* family. The results showed that recombinant Ts2631 endolysin may be effective in dealing with Gram-negative bacteria, such as *A. baumannii* and *P. aeruginosa*. EDTA and Ts2631 decreased all *Enterobacteriaceae* pathogens, including MDR *Citrobacter braakii*. There are two recombinant endolysins used for tackling MDR strains and persisters of *P. aeruginosa*. To produce recombinant Art-085 (Artilysin 085) and its improved homolog (Art-175), the sequence of SMAP-29 (sheep myeloid 29-amino acid peptide) was fused to the 5 end of KZ144's open reading frame. In comparison to KZ144, Art-085, and Art-175 penetrated the outer membrane, punctured the peptidoglycan layer, and degraded persisters and MDR *P. aeruginosa* rapidly and impressively. The cross-resistance between Art-175 and 21 therapeutically used antibiotics was also not observed (Briers, Walmagh, Grymonprez et al., 2014).

4.3 | Effectiveness of phage and engineered proteins against biofilms caused by superbugs

In a recent study by Son et al. (Son et al., 2021), four endolysins from *S. aureus* phages were screened for engineered properties. Lys109 was selected as a novel chimeric endolysin and demonstrated greater staphylolytic activity than its parental endolysins against staphylococcal biofilms and planktonic cells, as well as improved removal of *S. aureus* from steel and milk surfaces. In an attempt to inhibit antibiotic-resistant bacteria, Yuan et al. (Yuan et al., 2021) applied the endolysin of the phage vB_AbaP_D2. With an amphipathic helix, Abtn-4 showed a high activity against MDR Gram-negative strains, particularly *A. baumannii*, which was killed by Abtn-4 (5 μM) in 2 h. It also exhibited broad bactericidal properties against gram-negative and gram-positive bacteria, such as *P. aeruginosa*, *S. aureus*, *Enterococcus*, *K. pneumoniae*, and *Salmonella*. The Abtn-4 also showed killing activity against phage-resistant bacterial mutants in addition to its potential to decrease biofilm formation. The antibacterial activity of engineered peptides of phage lysins was studied by Thandar et al. (Thandar et al., 2016). MDR *A. baumannii* could be killed by the C-terminal amino acids of phage lysin PlyF307, referred to as P307 (there were >3 logs endolysins that showed increased lytic effect against MDR *S. aureus* when compared with their parental forms. P307 and P307SQ-8C (engineered form) showed high in vitro activity against *A. baumannii* biofilms. Additionally, peptides disrupted the bacterial membrane but did not kill human red blood cells or B cells and reduced MDR *A. baumannii* burden in skin infections of mice. Researchers in another study constructed a hybrid endolysin by swapping an enzymatically active domain endolysin with the endolysin that binds to the cell wall. As compared to its parental endolysin form, ClyC was a novel chimeric endolysin that demonstrated enhanced lytic activity against MDR *S. aureus*. Furthermore, ClyC effectively eliminated biofilms of MDR bacteria, including methicillin-resistant epidermidis, methicillin-resistant *S. aureus*, and *S. aureus* clinical isolates and was also effective against methicillin-resistant *S. aureus* without any toxic effects in an in vivo mouse infection model (Lee et al., 2021). Based on 21 different lysins with different activities, the PlyF307 lysin showed the greatest activity,

TABLE 1 A Summary of main studies that revealed the effectiveness of engineered phages, derived proteins, and enzymes against superbug bacteria.

References	Engineered enzyme or phage	Antibiotic resistant bacteria	Summary of result
Defraine et al. (2016)	Manipulated artilysin (Art-175)	MDR *Acinetobacter baumannii*	Art-175 had high bactericidal properties against all MDR clinical isolates
Schirmeier et al. (2018)	Art-175	Colistin-resistant *Escherichia coli*	Art-175 revealed a high antimicrobial cope with *mcr-1*-producing isolates
Blasco et al. (2019)	Purified ElyA1 and ElyA2 endolysins	Carbapenemase-producing *Klebsiella pneumoniae* isolates, MDR *A. baumannii*, and MDR *Pseudomonas aeruginosa*	Although ElyA1 showed antibacterial activity, ElyA2 had not antibacterial effect
Thummeepak et al. (2016)	Recombinant LysABP-01 endolysin	MDR and XDR *P. aeruginosa*, *A. baumannii*, and *E. coli* strains	LysABP-01 can degrade bacterial cell walls and has synergism with various antibiotics
Wu et al. (2019)	Recombinant Ply6A3 endolysin	MDR *A. baumannii*, *E. coli*, and methicillin-resistant *Staphylococcus aureus* isolates	Ply6A3 endolysin disrupted gram-negative and positive isolates
Lood et al. (2015)	PlyF307 endolysin	MDR *A. baumannii* isolates	Not only PlyF307 was able to significantly decline planktonic and biofilm of clinical isolates
Jasim et al. (2018)	Purified endolysin	MDR, XDR, and PDR *A. baumannii* isolates	Endolysin efficiently solved the problems of superbug isolates by degrading potently most XDR and PDR isolates
Dedrick et al. (2019)	Engineered three-phage cocktail	Drug-resistant *Mycobacterium abscessus*	Intravenous engineered lytic phage treatment efficiently killed the infectious *M. abscessus*
Son et al. (2021)	Engineered Lys109 endolysin	Drug-resistant *S. aureus*	Lys109 showed greater staphylolytic activity than its parental endolysins against staphylococcal biofilms and planktonic cells
Yuan et al. (2021)	Recombinant Abtn-4 endolysin	MDR *A. baumannii*, *P. aeruginosa*, *S. aureus*, *Enterococcus*, *K. pneumoniae*, and *Salmonella*	Not only Abtn-4 had the potential to decrease biofilm formation but also showed killing activity against phage-resistant bacterial mutants
Lai et al. (2015)	Recombinant LysA and LysB endolysin	Drug resistant and standard strain of *Mycobacteria* spp.	Both LysA and LysB were showed narrow spectra of antimicrobial activity against only *Mycobacteria* spp. via significant modification of the Mycobacterial cell shape
Deng et al. (2021)	Recombinant LysO78 endolysin	Antibiotics resistant *Klebsiella*, *Pseudomonas*, *Salmonella*, *Burkholderia*, *Shigella*, *Yersinia*, *A. baumannii*, *Chitinimonas arctica*, and *Ralstonia solanacearum*, as well as MDR *E. coli*	Catalytic residues of LysO78 exhibited broad-spectrum bacteriolytic activity against different genera and species
Thandar et al. (2016)	Engineered P307SQ-8C endolysin	MDR *A. baumannii*	P307SQ-8C revealed high activity against planktonic and biofilms form of bacteria
Basit et al. (2021)	Engineered RL_Lys endolysin	MDR *P. aeruginosa*, *K. pneumoniae*, methicillin resistant *S. aureus*, and *Salmonella*	RL_Hlys exhibited active effect against wide range of bacteria
Briers, Walmagh, Grymonprez, et al. (2014)	Engineered artilysins	MDR *P. aeruginosa* and *A. baumannii*	Artilysins was able to kill MDR isolates

TABLE 1 (Continued)

References	Engineered enzyme or phage	Antibiotic resistant bacteria	Summary of result
Ding et al. (2020)	Recombinant LysSE24 endolysin	MDR *Salmonella* strains	LysSE24 had antibacterial activity against 23 tested strains
Emslander et al. (2022)	Engineered phage	MDR *K. pneumoniae*, *Yersinia pestis*, and enteroaggregative *E. coli*	Engineered phage has therapeutic activity against MDR bacteria
Lee et al. (2021)	Recombinant ClyC endolysin	Methicillin-resistant Staphylococcus epidermidis, methicillin-resistant *S. aureus*, and MDR *S. aureus*	ClyC represented increased lytic effect against MDR *S. aureus* and biofilm of Gram positive bacteria
Chandran et al. (2022)	Endo88 (recombinant endolysin) and VAH88 (recombinant virion-associated peptidoglycan)	MDR *S. aureus*	Endo88 and VAH88 lysed antibiotics resistant bacteria and Endo88 was more efficacious than VAH88
Qin et al. (2022)	Engineered EATPs phage	MDR *P. aeruginosa* isolates	EATPs achieved impressive antibacterial function along with high safety
Plotka et al. (2019)	Recombinant Ts2631 endolysin	MDR *P. aeruginosa*, *A. baumannii*, and pathogens from the *Enterobacteriaceae*	Ts2631 endolysin could be effective to cope with gram-negative bacteria and EDTA/Ts2631 combination decreased all pathogens of the *Enterobacteriaceae* family
Briers, Walmagh, Puyenbroeck, et al. (2014)	Recombinant Art-085 and Art-175 endolysins	MDR *P. aeruginosa*	Art-085 and Art-175 passed the outer membrane and punctured the peptidoglycan layer as a result degraded persisters and MDR *P. aeruginosa*
He et al. (2020)	AIEgens–PAP-modified phage	MDR *P. aeruginosa*	AIEgens–PAP-modified phage also demonstrated high efficiency in the cure of skin wounds involved with MDR *P. aeruginosa*

Abbreviations: MDR, multidrug resistance; PDR, pandrug-resistant.

which was efficient enough to kill all MDR *A. baumannii* isolates tested (>5 log-unit reduction). As well as killing lethal *A. baumannii* bacteremia in mice, PlyF307 significantly declined planktonic and biofilm *A. baumannii* in vitro and in vivo (Lood et al., 2015). To save patients from the warnings of antibiotic resistance, researchers are manipulating phages and their enzymes to enhance their efficiency (Table 1). As a result of the above studies, engineered phages and enzymes possess increased antimicrobial properties, increased penetration of the cell wall, the ability to destroy biofilm, the absence of cross-resistance with antibiotics, and enhanced thermostability. Using this method can be effective in destroying superbug bacteria.

5 | SYNERGISTIC EFFECT OF ENGINEERED PHAGES, PHAGE PROTEINS, AND PHAGE ENZYME ALONG WITH OTHER ANTIMICROBIAL AGENTS

A combination of engineered endolysin and antibiotics was also effective against MCR-1 positive *E. coli*. EC340, an endolysin in *E. coli* phage PBEC131, has been engineered for improved outer membrane permeability and drug resistance. Using LNT113 as an engineered endolysin, colistin showed synergistic activity, while ciprofloxacin, tigecycline, meropenem, azithromycin, and chloramphenicol showed additive activity (Hong et al., 2022). P114 was assembled as a self-assembling peptide with P128 as a phage lytic enzyme into nanoparticles in 2022 to create an engineered endolysin. As compared to native P128, P128 nanoparticles (P128NANO) have shown an enhanced antimicrobial activity against methicillin-resistant *S. aureus*. Aside from its increased thermal (up to 65°C) and storage stability, P128NANO also damaged bacterial cell walls extensively (Dzuvor et al., 2022). A study was conducted involving truncated bacteriophage endolysin CHAPK and lysostaphin nanoparticles in conjunction with Poly (N-isopropylacrylamide) (PNIPAM) nanoparticles for the control of MRSA. Bacteriocins and endolysins from bacteriophages have both been shown to possess antimicrobial efficacy against drug-resistant bacteria, while PNIPAM polymer shows synergy with an enzybiotic cocktail consisting of CHAPK and lysostaphin as delivery systems (Hathaway et al., 2017). In another study, ElyA1 and ElyA2 endolysins, alone or combined with colistin, were evaluated for their antimicrobial activity against MDR gram-negative bacteria. The antibacterial activity of ElyA1 was

detected against carbapenemase-producing pneumoniae isolates, MDR *A. baumannii*, and MDR aeruginosa, but no antibacterial activity was detected in ElyA2. Combining ElyA1 with colistin reduced colistin toxicity and MIC, thereby increasing colistin's bactericidal activity both in vitro and in vivo (Blasco et al., 2019). Lu and Collins (Lu & Collins, 2009) developed an experimental bacteriophage that targets non-SOS gene networks, overexpressed proteins, and attack gene networks. As a result, φlexA3 suppressed the SOS network in *E. coli* as a result increased killing by quinolones and remarkably enhanced survival of infected mice. Moreover, the φlexA3 showed enhanced activity in killing biofilm cells, persister cells, and antibiotic-resistant bacteria, as well as a significant reduction in the number of antibiotic-resistant bacteria, and an excellent adjuvant function for aminoglycosides and β-lactams. The combination of engineered lysine (eAbEndolysin) with cecropin A was developed in 2022 as an antimicrobial peptide against MDR *A. baumannii*. As a result of fusing cecropin A with eAbEndolysin, the bactericidal activity of MDR was enhanced against *A. baumannii*. As a result of fusing cecropin A with eAbEndolysin, the bactericidal activity of MDR was enhanced against *A. baumannii*. As well as having no cytotoxic effect on cell lines and saving mice from systemic *A. baumannii* infection, eAbEndolysin also showed synergistic effects with azteronme, beta-lactam antibiotics, ceftazidime, and cefotaxime, and an additive effect with imipenem and meropenem (Islam et al., 2022). Using Crotalicidin tag (15-34) as antimicrobial peptide and phage Endolysin T5, a novel recombinant endolysin peptide, EndoT5-Ctn (15-34), was developed. MDR *P. aeruginosa* was treated with EndoT5-Ctn (15-34) and shown to have antibacterial activity (Huynh et al., 2021). According to Park et al., recombinant endolysin LysECP2 combined with aromatic compounds in essential oils is effective against *E. coli*. As a result of the synergistic activity of recombinant LysECP26 with thymol or eugenol, the minimal inhibitory concentration of recombinant endolysin was four times lower when it was applied in combination with either of these compounds (Park et al., 2021). A lysin engineered by fusion of LysMK34 with Cecropin A, a strong antimicrobial peptide, was developed by Abdelkader et al. (Abdelkader et al., 2022) to combat *A. baumannii* infections. This engineered lysin (eLysMK34) shows improved antibacterial activity compared to the parental lysin, and was highly effective against MDR, XDR, and colistin-resistant gram-negative isolates. The antimicrobial activity of cationic carbosilane (CBS) dendrimers and the combination of CBS dendrimers with recombinant endolysin was investigated in 2022 against planktonic and biofilm *P. aeruginosa*. Even though recombinant endolysin and CBS mixtures are ineffective against Gram-negative bacteria when applied without cationic CBS dendrimers, they provide a synergistic effect that allows penetration through the membrane through a deterioration of the peptidoglycan layer (Quintana-Sanchez et al., 2022). Thus, the above studies suggest that the combination of engineered phages and enzymes with antibiotics, nanoparticles, antimicrobial peptides, bacteriocin, and chemical compounds could be a new strategy to fight pathogens that threaten humans.

6 | CONCLUSION

In today's world, molecular biology, genetic engineering, and chemical engineering techniques are rapidly advancing to produce safe and novel antimicrobial agents that follow natural bacterial viruses. Several of the limitations of phage treatment have not been addressed on a large scale to address antibiotic resistance, which has been a global public health problem for decades. To combat superbug bacteria that give the lucky chance to produce all necessary characteristics, using safety phage products constructed by genetically manipulated enzymes and phages is an appealing solution. Compared to whole phage particles, synthetic phage particles and engineered enzymes are better at penetrating tissues, have a wider antibacterial spectrum, are less prone to bacterial resistance, reduce biofilm formation, and are less immunogenic. Superbug bacteria can also be more effectively and effectively controlled by synthetic phages and engineered enzymes. As a result of synthetic molecular and biology engineering methods, it is possible to produce phages and enzymes on an uncomplicated basis, making the purification and production of these products on a large scale not far from the well-developed pharmaceutical technologies. Phage engineering has achieved great success in dealing with antibiotic-resistant bacteria, despite the fact that the study of engineered enzymes, phages, and proteins is still in its infancy. There are still many limitations with clinical trials of engineered enzymes and phages, especially in patients with superbug bacterial infections, despite all the successes they have achieved in laboratory conditions and animal models. It would therefore be appropriate to conduct a series of safety evaluations of engineered enzymes and phages and large-scale clinical trials, because these materials are regarded as a window of hope for the future of mankind due to their strong effectiveness against superbug bacteria.

AUTHOR CONTRIBUTIONS

Mahshid Badakhshan Boroujeni: Writing original draft preparation. **Samane Mohebi**: Conceptualization; writing original draft preparation. **Azam Malekian**: Writing original draft preparation. **Seyed Sadegh Shahraeini**: Investigation and editing. **Zahra Gharagheizi**: Writing original draft preparation. **Shaghayegh Shahkolahi**: Investigation; writing original draft preparation. **Rezvaneh Vahedian Sadeghi**: Writing original draft preparation. **Mahin Naderifar**: Writing review and editing. **Majid Reza Akbarizadeh**: Investigation and editing. **Simin Soltaninejad**: Writing original draft preparation. **Zahra Taati Moghadam**: Conceptualization. **Majid Taati Moghadam**: Conceptualization; writing original draft preparation; project administration. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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