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Phage-Derived Enzymes as alternative therapeutics against antibiotic resistant pathogens

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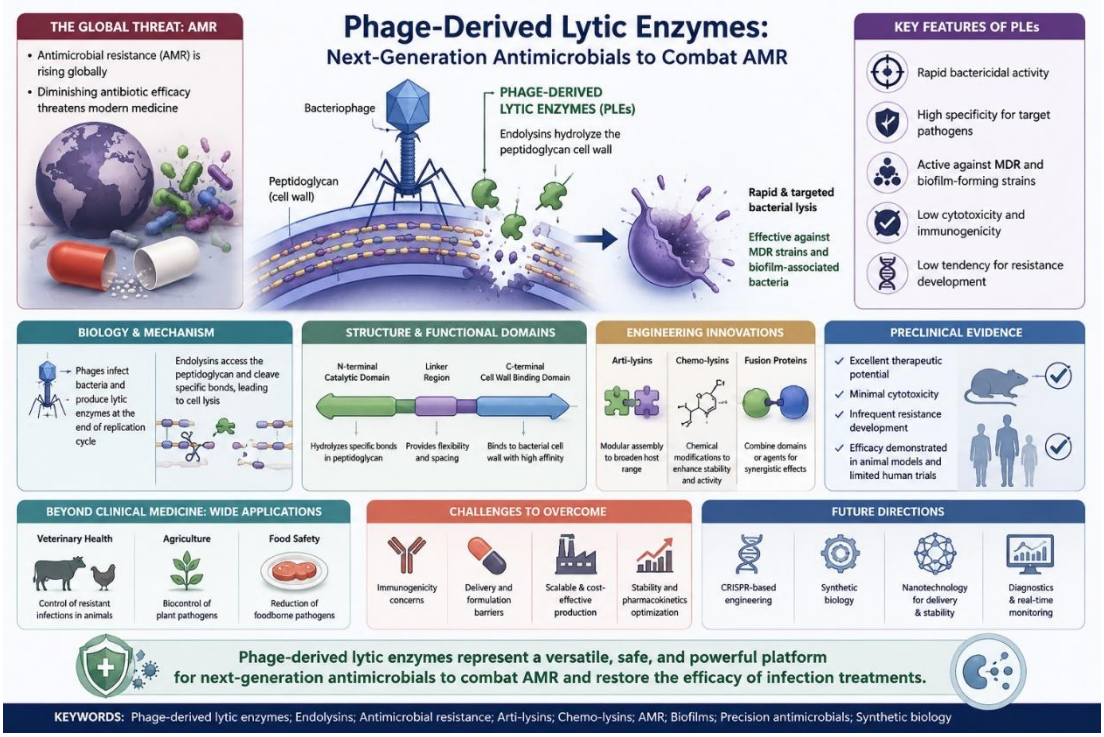
Abstract

Antimicrobial resistance is a globally rising threat to health, diminishing antibiotic efficacy and threatening contemporary medical treatments. As part of creative solutions, page derived lytic enzymes (PLEs), especially endolysins, to antibiotics. They hydrolyze the peptidoglycan bacterial cell wall allowing quick and targeted destruction of pathogens, including multidrug resistant (MDR) and biofilm-related strains. This review discusses the biology and mechanisms of PLEs, their functional domains, bactericidal specificity, and engineering innovations including arti-lysins and chemo-lysins that increase efficacy against Gram positive as well as gram negative bacteria. Preclinical trials have shown excellent therapeutic potential, with minimal cytotoxicity, Infrequent resistance development and safety conformed in animal and limited human trails. applications outside clinical medicine, including veterinary health, agriculture and food safety, demonstrate the wide potential of PLEs as sustainable antimicrobial agents. But there is still much to overcome in terms of immunogenicity, delivery methods and bulk productions. Future directions include combining CRISPR-based engineering, synthetic biology, nanotechnology and diagnostics innovations to increase enzyme stability, expand host range, and enhance pharmacokinetics profiles. Together, this review highlights the potential of phage - derived lytic enzymes as next generation therapeutics to combat AMR and regain efficacy in infection.

Graphical Abstract



Introduction

Antimicrobial drugs are the backbone of contemporary medicine, but the

evolution and dissemination of drug-resistant microorganisms jeopardize our capacity to treat endemic infections and to conduct life-saving interventions like cancer chemotherapy, caesarean section, hip replacement, organ transplantation, and other major surgeries. The rise in antibiotics resistance worldwide constitutes a serious challenge since it reduces the effectiveness of widely used antibiotics against prevalent bacterial infections (1). Antimicrobial resistance (AMR) is among the most urgent world public health and development issues. The 2022 Global Antimicrobial resistance and Use surveillance System (GLASS) report indicates shocking rates of resistance among key bacterial pathogens (2) Median resistance rates in 76 countries were reported to be 42% for third-generation cephalosporin-resistant Escherichia coli and 35% for methicillin-resistant Staphylococcus aureus. For E. coli in one out of five cases, reflected decreased susceptibility to typical antibiotics like ampicillin, co-trimoxazole more challenging to treat. Beyond humans, drug-resistant infections also impact animals and plants, lowering farm productivity and endangering worldwide food safety overuse and abuse of antibiotics over the last decade have hastened the evolutions of resistant bacteria, driving us to possible post antibiotic era," when previously insignificant bacterial infections could again pose a threat to life" (levy et al., 2004) (3). To this end, global health organizations are calling urgently for innovative approaches to counteract antibiotic resistant an prevent the spread of resistance (4) In this regard, bacteriophages and their lytic enzymes (lysins) are an encouraging area of exploration. In particular, one of the most encouraging substitutes or complements to traditional antibiotics is phage-derived lytic enzymes (PLEs).Other phage-encoded enzymes that have antibacterial activity (e.g. polysaccharide depolymerases that breakdown bacterial capsules, biofilms and outer membrane Lippopolysaccride of gram -negative bacteria are also being studied)(20) .phage lytic enzymes (PLEs)have at least one domain that cuts the peptidoglycan ,which is main structural element of bacterial cell wall (5). The peptidoglycan sacculates encapsulate the bacterial cytoplasmic membrane, which confers mechanical strength the structure is normally associated with osmotic lysis (6). For most tailed phages, penetration of rigid cell wall is crucial in two phases of the infection cycle: (1) to introduce viral nucleic acids into host cells and (2) to release progeny virions after replication. PLEs responsible for entry attack the cell wall from outside and are virion particle packed. thereby termed virion-associated lysins (VALS). That are responsible for progeny release are produced inside host cells and subsequently target the cell wall from inside, termed endolysins. Although 'endolysins' usually drops down to lysins, likewise VALs are simply referred to as virion-associated peptidoglycan hydrolases (VAPGH), Tail-associated murky tic enzymes (TAME), tail associated lysins (Tal), exo-lysins, or structural lysins. Therapeutically, recombinant PLEs are required to exhibit efficient killing activity in sophisticated biological media like animal tissues, mucosal membranes blood, and other body liquids. In addition, to maximizing antimicrobial activity, PLEs are likely to elicit host immunological responses that could neutralizing antibodies and unwanted side effects. Encouragingly, past safety studies -not least number of those conducted in humans -have shown no severe adverse effects and negligible interference from anti PLE antibodies. However, host immune responses always need to be taken into account, targeted interventions could be required to enhance the therapeutic value of PLEs further (7).

Biology and mechanism of phage-derived lytic enzymes:

Phages are simple but extremely varied non-living biological structures made up of DNA or RNA covered in a protein capsid. As natural parasitic bacteria, phages cannot reproduce by themselves and are ultimately reliant on bacterium host for survival. Phages usually attach to certain receptors on cell surface of bacteria, inject genetic material into host cell, either integrates this material into the bacterial genome (so-called 'temperate' phage) and vertically reproduce from mother to daughter cells, or take over the bacterial replicate machinery to produce the next phage generation progeny and kill the cell (so-called 'lytic' phages) (8). After attaining a phage progeny

critical mass, ranging from few to more than 1000 viral particles, depending on the conditions, the lytic proteins get activated and hydrolyze the peptidoglycan cell wall, liberating new phage to repeat the lytic cycle. Enzymes have been divided into two broad functional categories: Hollins, which dig holes in the bacterial cell membrane, and endolysins, virion cleave the peptidoglycan cell wall from the inside out to allow virion release as shown in figure no.1. Some phages also use virion-associated peptidoglycan hydrolases (VAPGHs) for primary entry and depolymerases to dismantle polysaccharide capsules or O-antigen chains for surface penetration. Phage-derived lytic enzymes also referred to as endolysins, are becoming powerful prospects for replacing traditional antibiotics, especially resistant infections (9). (Show in Figure 1)

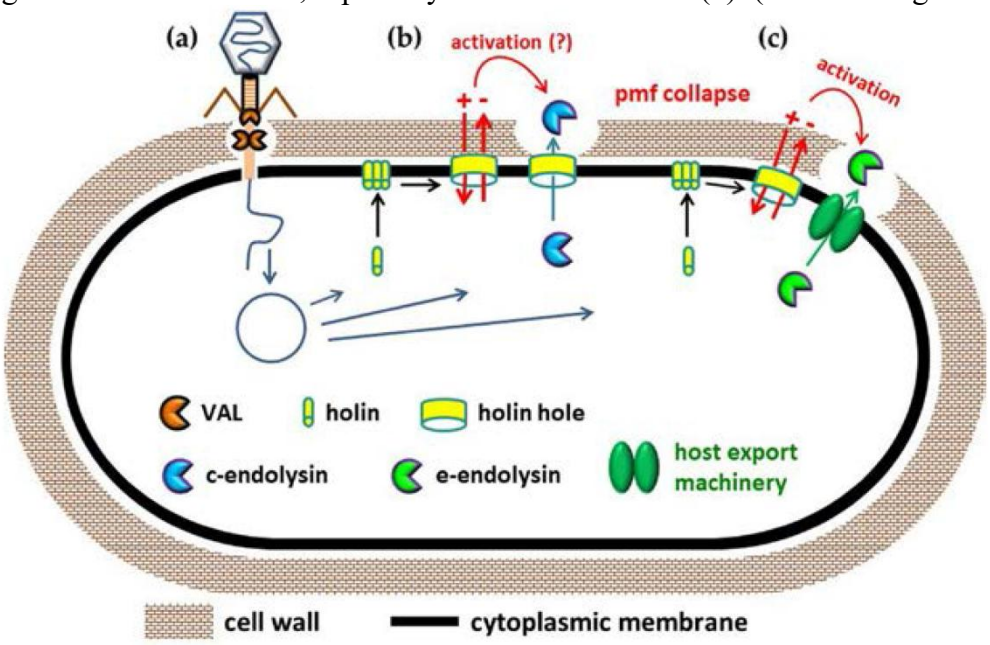


Figure No. 1: São-José, C. (2018). Engineering of Phage-Derived Lytic Enzymes: Improving Their Potential as Antimicrobials. *Antibiotics*, *7*(2), 29. https://doi.org/10.3390/antibiotics7020029 (10)

In gram positive bacteria like staphylococcus aureus, endolysins tend to have a modular design, usually consisting of one or multiple enzymatically active domains (EADs) and accompanying specialized cell wall binding domains (CBDs) which provide high efficacy as well as impressive target strain specificity (11). They have catalytic arsenal consisting of glycosidases, amides and endopeptidases, each of which cleaves a different bond within the peptidoglycan, thus directly breaking down the bacterial cell wall instead of just inhibiting its formation (12). One of the most illustrative examples is broad-spectrum staphylococcal endolysins Ly SYL, which not only killed planktonic cells but also possessed high biofilms-destroying capability, including against metabolically inactive persisted cells that are normal resistant to antibiotics (13). Conversely, Gram-negative cells pose another hurdle because of outer protective membrane, which shields direct access of the lysins to the peptidoglycan. Recent engineering efforts have addressed this by creating art-lysins and fusions proteins with cationic or membrane-active peptides, signal-anchor-release (SAR) motifs, or employes external permeabilizers like EDTA and polymyxins for entry thereby enhancing bactericidal potency considerably (14). Quantitative studies also support these reports: engineered lysins like Lys1S-L9P lowered E. coli loads by over 5-log CFU in one hour at micromolar concentrations and staphylococcal lysins like CHAPK-SH3B reached 3.9 µm/ml MICs and up to 4-log biofilm viability reductions without detectable resistance emergence. Together these developments emphasize that phage-derived lytic enzymes represent a mechanistically different and resistance-capable alternative to antibiotics but also identify current challenges, including in vivo stability, immunogenicity, delivery systems, and cost of productions, which first need

to be resolved before their maximum therapeutic potential can be harnessed. For facilitating the release of their progeny, double-stranded DNA phage cause provokes lysis of bacterial host, which is essentially achieved by breakdown of peptidoglycan. This polymer is a critical component of bacterial cell wall, and breaking particular bonds in three-dimensional network causes death in bacteria primarily by osmotic shock. The principal phage molecule responsible for peptidoglycan hydrolysis is lysin (also called endolysin) (15) lysins are secreted towards their polymeric target as shown in figure no.2, typically with the help of another type of protein, the Hollin, which form pores in plasma membrane and therefore permit leakage of lysin to periplasm. Phage products co-operate in inhibiting the cell wall in some of its specific contexts., lysins B remove arabino-muco-Lyle outer layer of mycobacteria and their relatives (16) (e.g. Rhodcoccus and corynebacterium). (Show in Figure 2)

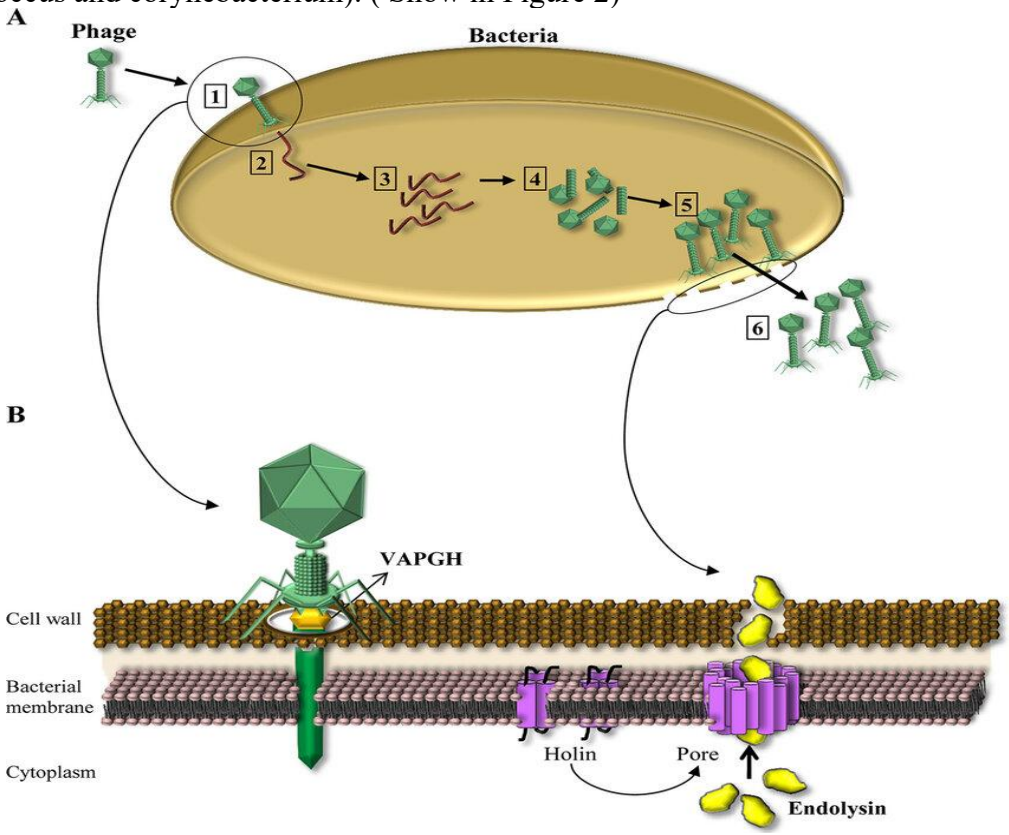


Figure No 2: Gutiérrez, Diana & Fernández, Lucía & Rodriguez, Ana & García, Pilar. (2018). Are Phage Lytic Proteins the Secret Weapon to Kill Staphylococcus aureus?. mBio. 9. e01923-17. 10.1128/mBio.01923-17.

Additionally in certain Gram-negative bacteriaG2, efficient lysis also requires the simultaneous involvement of other phage products that are known as spanins. This shows us the large amount of genetic material invested by phages to breach the obstacles that the bacterial cell walls pose. Along with application of intact phage particles as drugs against bacterial infections (so-called phage therapy), recent research also indicates artificial reusing some of the products of phage life cycle, like lysins, as antimicrobials. The principle is quite straightforward: the exogenous addition purified lysins to a permissive bacterium would result in bacterial lysis to a permissive bacterium would result in bacterial lysis whenever the lysin degrades the peptidoglycan. The process is simple in case of gram-positive bacteria G1, and the therapeutic activity of enzybiotics against G1 has been completely experimentally proven. The most significant features making enzybiotics suitable to be engineered as therapeutics are (i) some specificity towards the initial bacterial host and closely related bacteria which would avoid regular microbiota from being damaged or alternatively, the potential of broad-range lysins, if necessary; (ii) a lesser like hood of including the emergence of resistant bacteria ,which is hypothesized to result from the essential character of highly

conserved peptidoglycan ; (iii)the hope that neither harmful immune reactions nor in vivo production of neutralizing antibiotics will take place, perhaps as consequence of phage and their enzymes – being present among human regular microbiota and the rapid mechanism of action assumed to prevent antibody neutralization in vivo(17). Additionally, lysins can be subjected to protein engineering approaches. Generally structural organization of lysins consists of one enzymatically active domain (EAD) in combination with cell wall binding domain (CWBD) or occasionally, simply an EAD (18). Hence, synthetic biology approaches such as designing entirely novel lysins consisting of varied modules as building blocks, have been found feasible. These approaches allow to engineer and make customized antimicrobials upon the integration of a variety of functions of interest within one protein. Such functions of interest can be, apart from catalytic one against peptidoglycan meshwork, higher stability within complex or more generally, a certain tropism stability within complex more generally a certain tropism towards a specific component of bacterial surface or some other macromolecules, such as cellulose. The above engineering strategies have overcome the proposed inability of lysins to permeate the outer membrane of G2.

Various types of synthetic lysins have been designed to that effect. Among them are so-called art-lysins, which constitute lysins joined with various types of membrane-permeability peptides, the lysopines, which lysins coupled to elements from bacteriocins that allow recognitions of the bacterial surface and importation into Periplasm and endolysins, the lysins coupled to phage receptor-binding proteins have intrinsic bactericidal activity against g1 and g2. This activity was initially observed for T4 phage lysozyme and a number of pseudomonas aeruginosa phage lysins (19). This novel property was ascribed to non-enzymatic mechanisms, previously seen in partially denatured hen egg white lysozyme and depends on existence of antimicrobial peptides-like subdomains within such lysins, typically at c-terminal position, it was recently proposed that such (AMP) units are common in lysins from phage infecting G2 and they could act in concert in host lysis by establishing an extra affinity for the OM, due to their high net charge, relative to the negatively charged Lippo polysaccharides, and could contribute to lysis itself by having a membrane-destabilizing effect. Yet to what extent this character is widespread remains to be examined yet correctly, and thus its actual functional and evolutionary significance remans largely unknown. Of interest, such AMP-like motifs were used successfully to engineer AMPs that were functionally independent. To reveal the true evolutionary significance of these AMP-like motifs, as well as other lysins characteristics, like their domain composition, in this study, a bioinformatic strategy involving the analysis of broad set of lysin was implanted. There have been a number of precedents for use of homology-based analysis of putative lysin sequences that have led to the systemic understandings of the co-evolution of phage lysins and their hosts.The current work attempted to broaden the image with the newly available data, and to give solutions to the new questions raised by literature for lysin engineering. Thus, on the basis of present knowledge and genomic data available, we have assembled and built a comprehensive sequence database of phage lysin. Further analysis of data comprised (I) a preliminary survey of database structure, (ii) cross-referencing information replaced into database to verify differential distribution of different domain families and their architectural combinations within diverse bacterial groups and (iii)screening of readily computable physiochemical characteristics (net charge, hydrophobicity) along amino acids sequences to investigate widespread, related differences between groups. The findings here in will enhance our awareness of lysins specificity, diversity and contribute towards future drug design endeavors based on phage products (20)

Therapeutic applications against AMR bacteria:

Gram negative bacteria are especially challenging to treat owing to the possession of outer cell membrane, which has an adverse impact on permeability of antibiotics and is a critical determinant of intrinsic antibiotic resistant. Gram-negative

rods are responsible for several types of infections, such as intra-abdominal infections, UTIs and nosocomial infections. More than 25% of all pathogen related nosocomial infections are caused by bacteria with p. aeruginosa, K. Pneumonia and E. coli infections. This making gram-negative infections a continuing public health threat that, together with antibiotic resistance, increases the necessity for possible treatments. Aside from this antibiotic resistance by virtue of their cell envelope structure, Gram-negative bacteria also have several other mechanisms of antibiotic resistance. Such as mechanism include restriction of uptake of drug, alteration of drug to target, drug inactivation, and active drug efflux. The therapeutic usefulness of novel antimicrobial used to treat resistant bacteria is being undermined. For instance, cefiderocol with its new catechol-based iron transport mechanism for targeting the B-LACTAM antimicrobial agents to cell envelope of multidrug resistant Gram-negative bacteria can be assailed by a broad array of resistance mechanism. This includes new B-lactamases, efflux pumps, porin mutation, and receptor mutation of siderophores, as well as altered penicillin-binding proteins. Hetero resistance in strains of Acinetobacter Baumann also presents another challenge to antimicrobial therapy allowing a subpopulation of resistant organism to develop during the treatment and to share genetic information with susceptible population. The pathogens are categorized as multidrug resistant (MDR) extensively drug resistant (XDR) and pan drug resistant (PDR)Although European center for disease and prevention (ECDC) and centers for disease control and preventation (CDC) ended these terms as ;MDR was defined as acquired non susceptibility to at least 1 in 3 or more antimicrobial categories, XDR was defined as non-susceptible to at least 1 agent in all but 2 or fewer antimicrobial categories, and PDR was defined as non-susceptibility to all agents in antimicrobial agents. The bacteria include there are; *A. Baumann, K. pneumonia* and *P. aeruginosa* etc. Phage therapy has been studied in recent researches involving animal models against various clinically important pathogens. Oral phage that was administered when mice were challenged with sepsis caused by gut-derived P. aeruginosa rescued 66.7% of mice as opposed to 0% those in control group. As single dose of phage along c. difficile administration was enough prophylaxis against infection in a hamster model of clostridium difficile- induced ileoceca is; post infection phage treatments rescued 11 out of 12 mice while control animals treated with c. difficile and clindamycin succumbed within 96%h. phage mixtures also substantially inhibited *C. difficile* growth in vitro and restricted proliferation in vivo in hamster model. Single intraperitoneal delivery of a single phage strain was able to cure 100%, extended spectrum of beta-lactams producing *E. coli* and imipenem-resistant *P. aeruginosa*. Phage in cocktails have also been employed to cure antibiotic-resistant P. aeruginosa infections of the skin, lungs, and gastrointestinal tract in animal models. Further resistant *E. coli* O25:H4ST131, *vibrio parahaemolyticus, S. aureus* and *A. baumannii*. Antibiotic resistance genes that code for bacterial resistance to most antibiotics, such as beta-lactams, aminoglycosides, chloramphenicol's, and tetracycline, are threatening modern medical treatment of widespread in the environment. The dissemination of antibiotics resistance genes presents a special threat in that several antibiotics diminish their effectiveness against frequent infections, especially the challenging to treat nosocomial infections caused by ESKAPE pathogens *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter spp.).* The majority of phages are infective only to bacterium that bears their complementary receptors, thus effectively dictating lytic phage host range. Host specificity in different phages, some being strain-specific while other have shown the ability to infect a variety of bacterial strains and even genera. Bacteria have developed many means to invade infections by lytic phages, and phages have no less remarkable variety of mechanism to overcome to this resistance. For the bacteria, this can involve receptor alteration or loss, as well as phage DNA integration into CRISPR/Cas system, while for the phage this can involve recognition of new or

modified receptors and anti-CRISPR genes. The most prevalent lytic phages found with human pathogens and gut microbiota in orders cardioviral, also known as 'tailed phage' that have double-stranded DNA genomes and microviridin, which do not have tail but are single-stranded DNA viruses. As opposed to lytic phages, lysogenic phages insert their genetic material into the bacterial chromosomes as endogenous prophage (less frequently the phage DNA can exist independently as plasmid but yet stably passed through bacterial generations). The bacterial lysogens then pass on the prophage with every cell division. Environmental stressors on the bacterial host have the potential to induce the lysogenic phage from the latent prophage form initiating a switch to lytic cycle and the release of phage progeny into the environment. Upon integrating their genetic material into bacterial genome, genes encoded by prophage become available for host transcription. As many as 18 prophages have been reported in a single bacterial genome, such as in the foodborne pathogen *Escherichia coli* O157:H7 strain *Ska*i, with up to 20% bacterial chromosomal material consisting of genes may be advantageous to bacterial host and may code for virulence factors (e.g. diphtheria toxins, Shiga toxins, and botulinum toxins), metabolic genes, and antibiotic-resistant genes. Phage biologists currently understand that phage lifecycles may range on a continuum between lytic and lysogenic with pseudo-lysogenic, chronic, and cryptic lifecycles as example of new classification. Traditional phage therapy is based on the use of strictly lytic phages, which are obligate in their bacterial hosts. Lytic phages for use in treatment are assembled into preparations referred to as 'phage cocktails' which are composed of several phages demonstrated to be in vivo efficacy against the target pathogen.

Pharmacological considerations of phage lytic enzymes:

Endolysins have predictable pharmacokinetics profile relative to intact bacteriophages due to their actions as protein-based enzymes without being replicated within the host. Their initial rapid bactericidal actions, which clears out significant bacterial loads within the host. Their initial swift bactericidal effect, eliminating substantial bacterial loads Hurs after injections, has been graphically confirmed in animal models (Wojciechowska et al., 2024). Pharmacokinetic dare in humans are not available and would serve to clarify in greater detail biodistribution patterns.in gram negative organisms, outer membrane presence restricts lysin action, a consideration that has led to the development of engineered forms conjugated with membrane-permeabilizing peptides to facilitate greater access to their target sites (2025) The immune system itself is capable of generating neutralizing antibodies against bacteriophages, which compromises their therapeutic efficacy in certain clinical circumstances (Sawa et al.,2024). In contrast there are few cases reports, which suggest that rapid bactericidal actions, ahead of being inactivated by immune system repeated dosing and chronic treatment in humans have, however, not been widely investigated, and there is degree of skepticism concerning their potential in being immunogens. To mitigate this, novel, designed lysins, with reduced immunogenic domains, have been designed in a bid to ensure minimal interference from the host immune system (Khan et al., 2024). Preclinical safety test verifies that lysins are only minimally cytotoxic and not toxic to maintain cells at therapeutics level. moreover, their limited sphere of action implies that commensal microbiota is not appreciably inactivated, a significant point of differentiation from traditional broad-spectrum antibiotics. Local and topical applications, e.g., in infected wound and in catheter-associated biofilm, have demonstrated broad safety margins. However, intra-venous and systemic treatment are clinically poorly substantiated, and broad-scale trials must firmly establish in humans their safety It is not developed to resistance against endolysins, as opposed to the traditional antibiotics. Some research has shown that after being exposed multiple times bacteria are unable to develop stable resistant mutants, mainly because lysins specifically target highly conversed peptidoglycan links essential for bacterial viability. Resistance, however, is very common occurrence in whole phage therapy and there is possibility for bacteria to modify receptors in order to escape attachment of phages.

Future directions:

Phage -encoded lytic enzymes exert their actions by hydrolyzing peptidoglycans from the peptidoglycan layer of bacterial cell wall, leading to rapid lysis of pathogens.in contrast to traditional antibiotics, these are extremely specific, do not destroy normal microbiota, are active against planktonic them as agents to against antimicrobial resistance (AMR), mainly in infections where biofilm formation or multidrug resistance compromises antibiotic efficacy.The future progressions of lytic enzymes treatment are inextricably connected with new biotechnologies. CRISPR-mediated engineering could be used to customize lysin genes, engineering specialized enzymes with broadened host ranges, higher levels of catalytic efficiency and greater stability. Synthetic biology approaches allow chimeric enzymes such as artilysins and chemo lysine to be engineered, which have the ability to translocate beyond the outer membrane of Gram-negative bacteria an area where natural lysins are commonly limited. Concurrently, novel delivery system, such as nanoparticles, hydrogels and inhalable one, will provide expanded therapeutic options. Another significant direction is diagnostic and screening technology convergence. Such methods as surface [salmon resonance (SPR) have ability to map enzyme-pathogens binding interactions in real time allowing for faster identification. Protein engineering strategies such as PEGylation and fusion to antimicrobial peptides are able to decrease immunogenicity and increase circulation timing blood. Beyond human medicines, there are applications of lytic enzymes in veterinary health, agriculture, and food security that could sustainable for antibiotics and limit transmission of AMR throughout ecosystem.

The continued escalation of antimicrobial resistance (AMR) necessitates the development of innovative antimicrobial strategies that extend beyond conventional antibiotics. Among the most promising alternatives, phage-derived lytic enzymes (PLEs), particularly endolysins, have demonstrated exceptional potential because of their rapid bactericidal activity, high specificity, low propensity for resistance development, and efficacy against multidrug-resistant (MDR) and biofilm-forming pathogens. Nevertheless, despite encouraging laboratory findings and preclinical investigations, the widespread clinical implementation of PLEs remains limited by several biological, technological, pharmacological, and regulatory challenges. Future research should therefore focus on integrating advances in molecular biology, synthetic biology, protein engineering, computational sciences, nanotechnology, precision medicine, and global One Health initiatives to maximize the therapeutic potential of these enzymes. Such multidisciplinary efforts are expected to transform phage-derived lytic enzymes into next-generation antimicrobial therapeutics capable of addressing the rapidly expanding global burden of AMR (Khan et al., 2024; Ahmed et al., 2024; Butt et al., 2024a; Butt et al., 2025a; Butt et al., 2025b; Butt et al., 2025c).

One of the foremost priorities for future research is the rational engineering of lysins with improved biochemical and therapeutic characteristics. Although naturally occurring endolysins possess remarkable catalytic efficiency against Gram-positive bacteria, their clinical performance may be limited by restricted host range, moderate thermal stability, susceptibility to proteolytic degradation, and relatively short systemic half-life. Advances in structural biology and computational protein engineering now provide opportunities to redesign enzymatically active domains (EADs) and cell wall-binding domains (CBDs) through domain swapping, site-directed mutagenesis, directed evolution, and structure-guided optimization. These strategies may produce enzymes with enhanced catalytic activity, broader antimicrobial spectra, improved environmental stability, greater resistance to proteases, and superior pharmacokinetic profiles. Simultaneously, engineering efforts should prioritize reducing immunogenic epitopes without compromising antibacterial potency, thereby improving long-term therapeutic applicability and repeated dosing regimens (Butt et al., 2025a; Butt et al., 2025c; Butt et al., 2026a; Butt et al., 2026b; Butt et al., 2026c; Butt et al., 2026d; Butt et al., 2026e; Butt et al., 2026f).

Synthetic biology represents another transformative frontier in the development of PLE-based therapeutics. Future investigations should focus on constructing multifunctional chimeric lysins that combine catalytic domains from different bacteriophages with antimicrobial peptides, membrane-permeabilizing motifs, receptor-binding proteins, depolymerases, and biofilm-disrupting enzymes. Such engineered molecules—including artilysins, lysocins, and chemo-lysins—have the potential to overcome the outer membrane barrier of Gram-negative bacteria, which remains one of the principal limitations of naturally occurring lysins. Rationally designed synthetic enzymes may therefore provide effective therapeutic options against highly resistant Gram-negative pathogens such as *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and carbapenem-resistant *Escherichia coli*. Continued optimization of modular enzyme architecture through synthetic biology will likely generate customizable antimicrobial platforms tailored to specific bacterial species, resistance mechanisms, and clinical applications (Jabeen et al., 2025; Rashid et al., 2026; Riaz et al., 2026a; Riaz et al., 2026b; Butt et al., 2026g; Butt et al., 2026h; Butt et al., 2026i; Butt et al., 2026j).

CRISPR-Cas technologies are also expected to revolutionize the future development of phage-derived therapeutics. Beyond their established role in bacterial genome editing, CRISPR systems may enable precise modification of phage genomes and lysin-encoding genes to optimize catalytic activity, alter substrate specificity, improve protein stability, and eliminate undesirable genetic elements. CRISPR-assisted engineering may further facilitate the generation of programmable lysins capable of selectively targeting bacterial strains carrying specific resistance genes or virulence determinants while minimizing collateral damage to beneficial microbiota. Such precision-engineered antimicrobials could become important components of personalized infectious disease management, reducing unnecessary antibiotic exposure and limiting the emergence of new resistance mechanisms. Integration of CRISPR engineering with synthetic biology and recombinant protein expression platforms may substantially accelerate the development of highly specialized lysin therapeutics suitable for diverse clinical scenarios (Fatima et al., 2026; Jabeen et al., 2025; Kamal et al., 2026; Naeem et al., 2026a; Butt et al., 2026k; Butt et al., 2026l; Butt et al., 2026m).

Artificial intelligence (AI) and machine learning are anticipated to become indispensable tools throughout every stage of lysin discovery, optimization, and clinical translation. Rapid advances in deep learning, protein structure prediction, molecular docking, molecular dynamics simulations, and large-scale genomic mining provide unprecedented opportunities to identify novel lysins from extensive bacteriophage sequence databases. AI algorithms may accurately predict enzyme stability, catalytic efficiency, substrate specificity, toxicity, immunogenicity, and pharmacokinetic behavior before laboratory validation, thereby substantially reducing experimental costs and accelerating drug development. Moreover, AI-assisted computational design can facilitate the generation of customized lysins optimized for individual bacterial pathogens, patient microbiome composition, infection sites, and resistance profiles. Such precision antimicrobial development aligns closely with emerging concepts of personalized medicine and intelligent therapeutic decision-making (Kamal et al., 2026; Naeem et al., 2026a; Naeem et al., 2026b; Naeem et al., 2026c; Faizi et al., 2026; Arif et al., 2026; Awais & Butt, 2026; Butt et al., 2026n; Butt et al., 2026o; Shah & Butt, 2026).

Future research should also prioritize the development of advanced drug delivery technologies capable of overcoming current pharmacological limitations associated with protein therapeutics. Although recombinant lysins exhibit rapid bactericidal activity, their therapeutic effectiveness may be reduced by enzymatic degradation, limited tissue penetration, rapid renal clearance, and inadequate localization at infection sites. Nanotechnology-based delivery systems—including

polymeric nanoparticles, liposomes, dendrimers, nanoemulsions, biodegradable hydrogels, injectable biomaterials, microneedles, and inhalable formulations—may substantially enhance enzyme stability, prolong systemic circulation, enable controlled release, and improve tissue-specific targeting. Surface functionalization of nanoparticles with pathogen-specific ligands or antibody fragments may further enhance selective accumulation at infected tissues while minimizing systemic toxicity. Such delivery platforms could significantly improve the treatment of chronic wound infections, pulmonary infections, catheter-associated biofilms, orthopedic implant infections, urinary tract infections, and other difficult-to-treat bacterial diseases (Butt et al., 2025a; Butt et al., 2026g; Butt et al., 2026h; Butt et al., 2026p; Butt et al., 2026q; Butt et al., 2026r; Riaz et al., 2026b; Ullah et al., 2026a; Ullah et al., 2026b).

Another major direction involves developing highly effective combination therapies that exploit the complementary mechanisms of multiple antimicrobial agents. Rather than functioning as standalone therapeutics, future lysin formulations may be combined with conventional antibiotics, bacteriophages, antimicrobial peptides, probiotics, quorum-sensing inhibitors, bacteriocins, depolymerases, and biofilm-dispersing enzymes to achieve synergistic antibacterial activity. Such combination strategies may reduce antibiotic dosage requirements, minimize toxicity, prevent resistance development, and improve eradication of persistent biofilm-associated infections. Comprehensive evaluation of synergistic interactions through systems biology, pharmacodynamic modeling, and clinical validation will be essential for designing optimized multidrug treatment protocols suitable for increasingly complex MDR infections (Ahmed et al., 2024; Butt et al., 2024a; Rashid et al., 2026; Riaz et al., 2026a; Riaz et al., 2026b; Butt et al., 2026f; Butt et al., 2026p; Butt et al., 2026q; Nawaz et al., 2026).

The successful clinical translation of phage-derived lytic enzymes will depend largely on comprehensive pharmacological characterization and rigorous clinical validation. Although numerous preclinical studies have demonstrated excellent antibacterial efficacy with minimal toxicity, large-scale human clinical trials remain limited. Future investigations should therefore establish standardized protocols for evaluating pharmacokinetics, pharmacodynamics, biodistribution, tissue penetration, metabolic stability, dosage optimization, immunogenicity, and long-term safety following repeated administration. Particular attention should be directed toward evaluating enzyme performance in vulnerable patient populations, including immunocompromised individuals, neonates, elderly patients, transplant recipients, and intensive care unit populations that experience disproportionately high burdens of multidrug-resistant infections. The incorporation of standardized clinical endpoints and harmonized reporting criteria across international clinical trials will further facilitate evidence synthesis and regulatory approval (Mahmood et al., 2026; Butt et al., 2026a; Butt et al., 2026b; Butt et al., 2026f; Arif et al., 2026; Nadeem et al., 2026).

In parallel, internationally harmonized regulatory pathways must be established to accelerate the commercialization of lysin-based therapeutics. Unlike conventional antibiotics, recombinant lysins represent biological therapeutics with unique manufacturing, quality-control, and stability requirements. Future research should therefore contribute to the development of standardized Good Manufacturing Practice (GMP) protocols, quality assurance procedures, batch consistency assessments, stability testing guidelines, potency assays, and regulatory evaluation frameworks specifically designed for enzyme-based antimicrobials. Collaborative efforts among academic institutions, biotechnology companies, pharmaceutical manufacturers, and international regulatory agencies will be essential to streamline approval processes while ensuring product safety, efficacy, and reproducibility. Economic evaluations examining cost-effectiveness, healthcare savings, and market accessibility should also accompany regulatory development to facilitate widespread clinical adoption (Khurshid et al., 2026; Butt et al., 2026g; Awais & Butt, 2026; Rehman et al., 2026;

Naeem et al., 2026b; Naeem et al., 2026c).

Another important future priority involves optimizing industrial-scale manufacturing technologies capable of producing highly purified recombinant lysins at commercially viable costs. Advances in recombinant protein expression, continuous fermentation, cell-free protein synthesis, downstream purification, and lyophilization technologies are expected to improve production efficiency while maintaining enzyme activity and structural integrity. Simultaneously, formulation research should focus on developing thermostable preparations with prolonged shelf life that remain effective under diverse storage conditions, particularly in low- and middle-income countries where antimicrobial resistance represents an increasing public health challenge. Scalable manufacturing processes combined with affordable production costs will ultimately determine the global accessibility of lysin therapeutics (Butt et al., 2025a; Butt et al., 2025b; Butt et al., 2026h; Butt et al., 2026n; Butt et al., 2026r; Butt et al., 2026s).

Precision medicine is expected to play an increasingly important role in the future application of phage-derived lytic enzymes. Integration of rapid molecular diagnostics, metagenomic sequencing, antimicrobial susceptibility testing, and artificial intelligence-assisted clinical decision-support systems could facilitate individualized enzyme selection according to pathogen identity, resistance genotype, virulence profile, microbiome composition, and patient-specific clinical characteristics. Such personalized therapeutic strategies would maximize antibacterial efficacy while minimizing unnecessary antimicrobial exposure and preserving beneficial microbial communities. Future diagnostic platforms integrating biosensors, microfluidics, real-time sequencing, and machine learning algorithms may enable rapid identification of susceptible bacterial strains, allowing clinicians to administer customized lysin formulations within hours rather than days (Kamal et al., 2026; Naeem et al., 2026a; Naeem et al., 2026b; Arif et al., 2026; Butt et al., 2026i; Butt et al., 2026o; Butt et al., 2026p; Nadeem et al., 2026).

Beyond human medicine, phage-derived lytic enzymes possess enormous potential across veterinary medicine, livestock production, aquaculture, agriculture, and food safety. Future investigations should evaluate lysins as sustainable alternatives to prophylactic antibiotics used in food-producing animals, thereby reducing selective pressure for antimicrobial resistance while improving animal health and productivity. In aquaculture, enzyme-based therapeutics may provide environmentally friendly approaches for controlling bacterial diseases without promoting antibiotic resistance in aquatic ecosystems. Similarly, agriculture could benefit from lysin-based biocontrol strategies targeting economically important bacterial phytopathogens, reducing chemical pesticide dependence and improving crop sustainability. Continued research should also examine the integration of lysins into food preservation systems, antimicrobial packaging materials, dairy processing, meat production, and fresh produce sanitation to reduce foodborne pathogens and improve consumer safety (Ahmed et al., 2024; Butt et al., 2024a; Riaz et al., 2026a; Riaz et al., 2026b; Ullah et al., 2026a; Ullah et al., 2026b; Butt et al., 2026q; Butt et al., 2026r; Shafeeq et al., 2026).

Future applications should likewise embrace the One Health concept by recognizing the interconnected nature of human, animal, environmental, and agricultural antimicrobial resistance. Surveillance systems integrating clinical microbiology, veterinary medicine, environmental monitoring, wastewater epidemiology, food safety, and genomic sequencing may provide comprehensive assessments of resistance transmission pathways while identifying opportunities for targeted lysin deployment. Environmental applications, including wastewater treatment, hospital effluent management, livestock waste remediation, and bioremediation of contaminated ecosystems, represent additional areas where phage-derived enzymes could contribute to limiting the dissemination of antimicrobial

resistance genes. Such multidisciplinary approaches will require close collaboration among microbiologists, environmental scientists, veterinarians, epidemiologists, food technologists, and public health authorities (Riaz et al., 2026a; Ullah et al., 2026a; Ullah et al., 2026b; Riaz et al., 2026c; Riaz et al., 2026d; Gulzar et al., 2026; Rehman et al., 2026).

The incorporation of multi-omics technologies will further strengthen future lysin research by providing comprehensive insights into host–pathogen interactions, bacterial adaptive responses, enzyme specificity, microbial ecology, and resistance evolution. Genomics, transcriptomics, proteomics, metabolomics, metagenomics, and epigenomics can collectively identify novel therapeutic targets while elucidating mechanisms underlying bacterial susceptibility and enzyme performance. Coupling these datasets with advanced computational biology and systems-level analyses will facilitate the rational design of optimized lysins capable of targeting diverse bacterial populations while preserving microbial ecosystem stability. Such data-driven approaches may also support predictive modeling of treatment outcomes and accelerate precision antimicrobial discovery (Butt et al., 2026b; Fatima et al., 2026; Butt et al., 2026o; Ullah et al., 2026a; Arif et al., 2026; Shah & Butt, 2026).

Future commercialization efforts should additionally consider socioeconomic accessibility, healthcare equity, and implementation in resource-limited settings. Although lysin-based therapeutics possess significant scientific promise, their global impact will ultimately depend on affordability, manufacturing capacity, healthcare infrastructure, clinician awareness, and equitable distribution. Collaborative public–private partnerships, international funding initiatives, technology transfer programs, and interdisciplinary educational efforts will therefore be essential for ensuring that these advanced biologics become accessible beyond high-income healthcare systems. Investments in translational research, industrial biotechnology, and international scientific collaboration will play decisive roles in transforming laboratory discoveries into globally available antimicrobial interventions (Khurshid et al., 2026; Rehman et al., 2026; Naeem et al., 2026a; Naeem et al., 2026b; Faizi et al., 2026).

In conclusion, the future of phage-derived lytic enzymes extends far beyond their current role as experimental antibacterial proteins. Continued advances in protein engineering, synthetic biology, CRISPR-based genome editing, artificial intelligence, nanotechnology, precision medicine, multi-omics integration, and One Health implementation are expected to redefine the landscape of antimicrobial therapy during the coming decades. With sustained interdisciplinary collaboration among microbiologists, clinicians, engineers, computational scientists, pharmaceutical industries, regulatory agencies, veterinarians, agricultural scientists, and public health organizations, lysin-based therapeutics have the potential to become a transformative class of precision antimicrobials capable of combating multidrug-resistant pathogens while preserving microbiome integrity and reducing global dependence on conventional antibiotics. If current translational, manufacturing, regulatory, and clinical challenges are successfully addressed, phage-derived lytic enzymes may ultimately emerge as one of the most impactful therapeutic innovations for mitigating the worldwide antimicrobial resistance crisis and safeguarding modern medicine for future generations (Khan et al., 2024; Ahmed et al., 2024; Butt et al., 2024a; Butt et al., 2025a; Butt et al., 2025b; Butt et al., 2025c; Rashid et al., 2026; Kamal et al., 2026; Mahmood et al., 2026; Butt et al., 2026a–j; Fatima et al., 2026; Jabeen et al., 2025; Riaz et al., 2026a–b; Naeem et al., 2026a–c; Khurshid et al., 2026; Shah & Butt, 2026; Arif et al., 2026; Awais & Butt, 2026; Ullah et al., 2026a–b; Rehman et al., 2026; Faizi et al., 2026; Nawaz et al., 2026; Shafeeq et al., 2026; Gulzar et al., 2026; Nadeem et al., 2026).

Conclusion:

Escalating antimicrobial resistance firmly indicates a need for novel therapies beyond generic antibiotics, phage -encoded lytic enzymes, specifically endolysins and their rationally designed Derivatives, are a novel category of antibacterial agents

characterized by specific targeting, prompt bactericidal actions and lower potential for resistance emergence. Their confirmed efficacy against multidrug-resistant pathogens, such as pathogens internalized in biofilms, make them very effective agents to fill gaps in current regimes of treatment.Development in protein engineering, synthetic biology, and nanotechnology are gradually transcending limitations such as outer membrane penetration barriers, immunogenicity and divert limitations, and CRISPR-mediated alterations as well as fusion constructions are progressively broadening their scope as therapeutic agent. Notably, lytic enzymes s safety and narrow bioactivity profile offer advantage over broad-spectrum antibiotics by maintaining the host medicine, agriculture, and food safety mirror their broad applicability in decreasing global AMR BURDEN. For full potential, continued cross-disciplinary research, clinical proof of the concept and scalable methods of production will play a crucial role. Ultimately, phage -derived lytic enzymes are a transformative potential to redesign antimicrobial treatment in post-antibiotic era.

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