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Bacteriophage Endolysin: A Powerful Weapon to Control Bacterial Biofilms

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Abstract

Bacterial biofilms are widespread in the environment, and bacteria in the biofilm are highly resistant to antibiotics and possess host immune defense mechanisms, which can lead to serious clinical and environmental health problems. The increasing problem of bacterial resistance caused by the irrational use of traditional antimicrobial drugs has prompted the search for better and novel antimicrobial substances. In this paper, we review the effects of phage endolysins, modified phage endolysins, and their combination with other substances on bacterial biofilms and provide an outlook on their practical applications. Phage endolysins can specifically and efficiently hydrolyze the cell walls of bacteria, causing bacterial lysis and death. Phage endolysins have shown superior bactericidal effects in vitro and in vivo, and no direct toxicity in humans has been reported to date. The properties of phage endolysins make them promising for the prevention and treatment of bacterial infections. Meanwhile, endolysins have been genetically engineered to exert a stronger scavenging effect on biological membranes when used in combination with antibiotics and drugs. Phage endolysins are powerful weapons for controlling bacterial biofilms.

Graphical Abstract

Keywords Bacteriophage · Endolysin · Bacterial biofilms · Antimicrobial agents · Pathogenic microorganisms

Abbreviations

GlcNAc	*N*-acetylglucosamine
MurNAc	*N*-acetylmuramic acid
CBD	Cell wall-binding domain
CD	Catalytic domain
EADs	Enzymatically active domains
kDa	KDalton
L-peptide	Linking-peptide
Biofilms	Bacterial biofilms

1 Introduction

Bacterial biofilms are structural communities of bacteria wrapped in an extracellular polysaccharide matrix composed of lipoproteins, and fibronectin, produced by the organisms [1]. Biofilm formation is part of a survival strategy for an organism to resist suboptimal environmental conditions such as limited nutrient availability or lethal antibiotic concentrations. In nature, in some industrial settings (e.g., fermentation and wastewater treatment), and humans and animals, most bacteria grow as biofilms attached to the surface of living or non-living objects, rather than in a planktonic manner [2]. Bacteria within biofilms have a strong affinity for nutrients and are highly resistant to exogenous substances, making them independent and resilient in micro-ecosystems. Once pathogenic microorganisms form biofilms, they become more resistant to antimicrobial drugs and the host's immune system, causing persistent infection

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in the body [1, 2]. Biofilms can contain up to 97% water. In addition to water and bacteria, biofilms may contain secreted macromolecules, adsorbed nutrients and metabolites, and lysed bacterial products. Therefore, biofilms contain various major biomolecules, such as proteins, polysaccharides, DNA, RNA, peptidoglycan, lipids, and phospholipids. The formation of a biofilm multicellular structure is a dynamic process that includes the stages of initial bacterial adhesion, biofilm development, and maturation; bacteria in biofilms exhibit different physiological and biochemical properties at each stage (Fig. 1).

The effect of antimicrobial agents is usually significantly reduced in biofilms, presumably owing to their inherent properties [5]. Studies have shown that bacteria enclosed in biofilms are 10–1000 times more resistant to antibiotics than those in the planktonic form [6]. While it is difficult to remove biofilms using antibiotics or common disinfectants, numerous studies have shown that phages and their endolysins have good lytic effects on biofilms.

Bacteriophage endolysins (endolysins) are enzymes encoded by double-stranded DNA phages that depend on host bacteria for their synthesis, and they are produced late in the lytic cycle [7]. Most phages rely on endolysins to hydrolyze the bacterial cell wall [8]. The hydrolyzing of the cell wall is essential for releasing the phage progeny. Endolysins can kill many types of bacteria including the pathogenic and antibiotic-resistant. Endolysin therapy has been proposed based on the continuous emergence and spread of drug-resistant bacteria. The antimicrobial effect of therapeutically applied endolysins applies only to Gram-positive bacteria and Gram-negative are resistant due to the presence of a protective outer membrane unless the endolysins are genetically modified to overcome it. In this review, we will provide an outlook on the practical application of endolysins in the context of their bacteriolytic mechanism and removal of biofilms.

2 Structure and Bacteriolytic Mechanism of Phage Endolysins

Phage lysins of Gram-positive bacteria usually have a C-terminal cell wall-binding domain (CBD) that determines the specificity of cell wall-binding and an N-terminal catalytic domain (CD) that determines the catalytic activity of the enzyme (Fig. 2). However, the CD of Gram-negative bacteriophage lysins (e.g., *Pseudomonas aeruginosa* phage lysin) is generally located at the C-terminus, whereas the CBD is located at the N-terminal end [9, 10]. Some endolysins possess two or even three catalytic domains and a binding

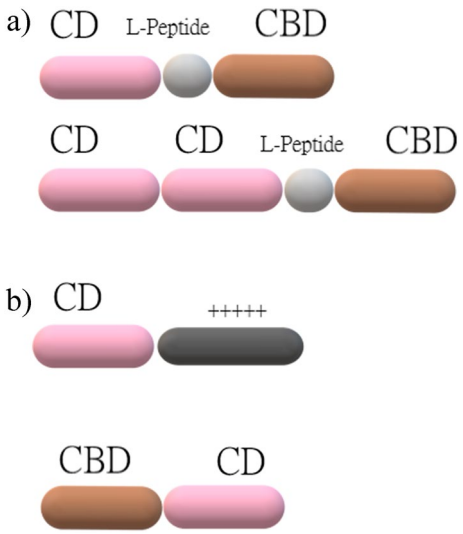

Fig. 2 The structural domain of endolysins. **a** Structural domain of Gram-positive endolysin: CD is an N-terminal catalytic domain, CBD is a C-terminal cell wall-binding domain, **b** Structural domain of Gram-negative endolysin: CD is generally located at the C-terminus, '++++' represents 2–3 possible catalytic structural domains

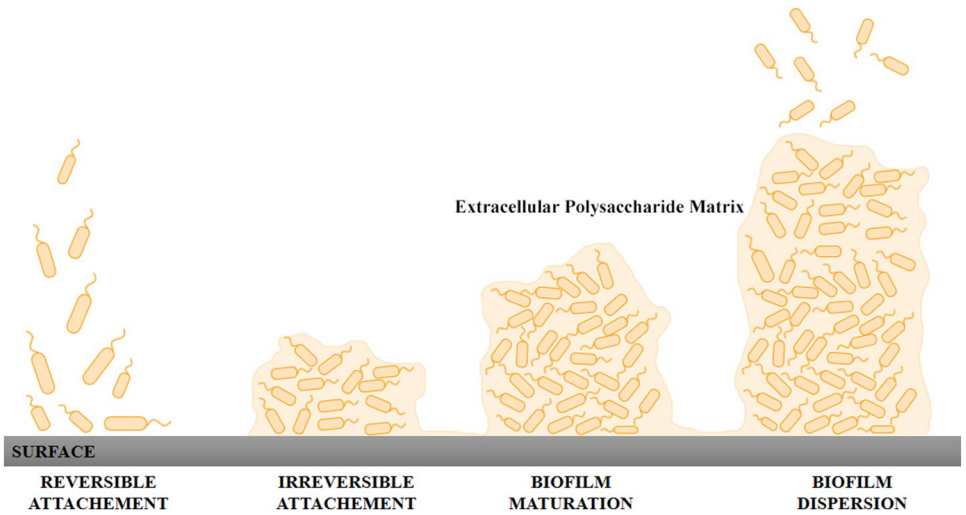

Fig. 1 Process of biofilm formation. [3, 4] Adapted from Maunders et al.

domain [11], such as the lytic enzyme HydH5 encoded by a *Staphylococcus aureus* phage Φ11, which possesses two catalytic domains and no conjugation domain [12]. Yang et al. [13] found that some endolysins have a separate spore-binding domain in their structure, which can recognize specific species of bacterial spores.

Endolysins mainly act on peptidoglycan peptides and glycosidic bonds (Fig. 3). Depending on the site of action of endolysins, they can be classified as endolysins/muramidase, which acts on the β-1,4-glycosidic bond of the glycan backbone in the cell wall; endopeptidases, which act on the polypeptide chain; or amidases, which hydrolyze the amide bond between the glycan backbone and polypeptide chain [14]. Because the amide linkage of peptidoglycan and the β-1,4-glycosidic linkage between aminosaccharides tend to be conserved among bacterial species [15], amidases have a broader cleavage spectrum. Moreover, peptidoglycans in bacterial cell walls are more conserved and less likely to be resistant to phage endolysins. Therefore, phage endolysins have great potential as antimicrobial agents.

Gram-positive and Gram-negative bacteria have different cell envelope structures (Fig. 4). The cell envelope in Gram-negative bacteria has a thinner peptidoglycan layer, an outer membrane and bacterial capsule or mucus layer covering the outer membrane. This makes it more difficult for endolysins to lyse Gram-negative bacteria from outside. Therefore, there are usually differences in the structure of endolysins that target Gram-positive and Gram-negative bacteria. Gram-positive endolysins have evolved to utilize a modular design in which catalytic activity and substrate recognition are performed by two different types of functional structural domains called CBDs and enzymatically active domains (EADs), respectively [10, 18]. EAD confers the catalytic mechanism of the enzyme (i.e., cleaves specific bonds within the bacterial peptidoglycan). However, endolysins with CBDs target proteins to their substrates and keep CBDs tightly bound to cell wall fragments after cell lysis, thereby preventing diffusion and disrupting the surrounding intact cells that have not yet been infected by phages [19]. In contrast, the outer membrane of Gram-negative bacteria can prevent such collateral damage by restricting endolysins from entering the peptidoglycan layer from the outside, which may explain why endolysins from phages infecting Gram-negative host bacteria are predominantly small single-domain globular proteins (molecular weight between 15 and 20 kDa) that usually do not have a specific CBD module [9]. Such endolysins may perform better as enzymes (aiding multiple catalytic reactions during cell lysis) than endolysins of Gram-positive bacteria, which bind to a site with a very low release rate [20]. Nonetheless, there are exceptions, such as the endolysins of *P. aeruginosa* (Gram-negative bacteria) phages, KZ144 and EL188, with modular structures of N-terminal CBD and C-terminal EAD. Both KZ144 and EL188 have a modular structure consisting

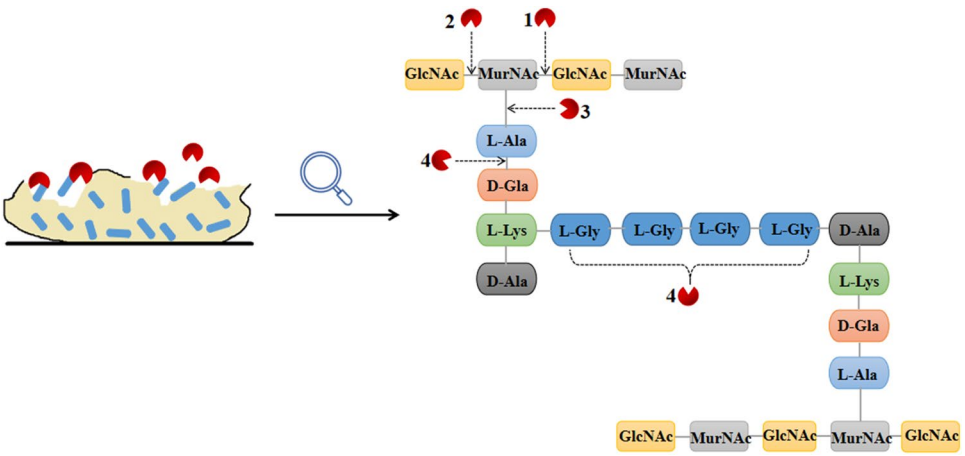

Fig. 3 Binding sites for endolysins. GlcNAc. N-acetylglucosamine; MurNAc. N-acetylmuramic acid [15], 1. MurNAc enzyme, 2. Endo-β-N-acetylglucosaminidase, 3. N-acetylcytidyl-L-alanine amidase; 4. Peptide chain endonuclease, amidase [16, 17]

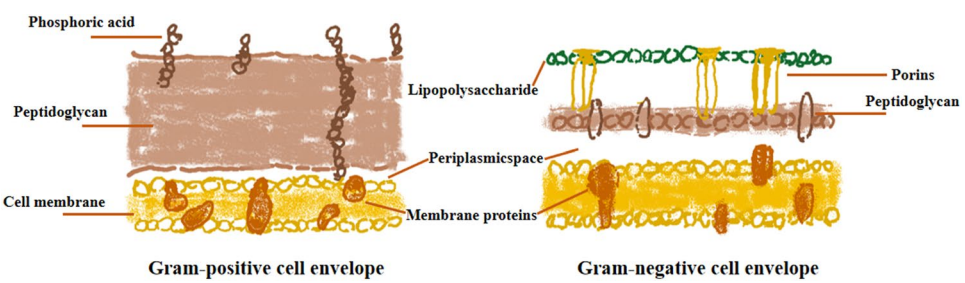

Fig. 4 Differences in cell envelopes between Gram-negative and Gram-positive bacteria

of an N-terminal substrate-binding domain and a predicted C-terminal catalytic module, a property previously only demonstrated in endolysins originating from phages infecting Gram-positives and only in an inverse arrangement. Both binding domains contain conserved repeat sequences, consistent with those of some peptidoglycan hydrolases of Gram-positive bacteria [9].

Shen et al. [21] suggested that there are two main mechanisms by endolysin PlyC act: (1) direct lysis of host bacteria and (2) degradation of extracellular matrix components of the biofilm, which exposes the bacteria. Based on the available results, the following speculation was made regarding the mechanism of biofilm removal by phages or their endolysins: the polysaccharide on the outer surface of the bacteria is known to be the main component of the extracellular matrix of the biofilm, and these "smart" phages recognize and degrade the polysaccharide as the main component by producing polysaccharide lysins. The biofilm structure is then destroyed and the intrinsic bacteria are exposed. Once inside the biofilm, the phages invade and lyse the host bacteria, releasing more progeny phages and accelerating the lysis of the bacteria while also preventing the formation and maintenance of the new biofilm. Notably, bacteria within biofilms often exhibit multidrug resistance. The ineffectiveness of antibiotics against biofilms and their large-scale use is contributing to the growing problem of antimicrobial resistance. Phages and their endolysins can potentially be applied to remove biofilms.

3 Native Phage Endolysins Clear Biofilm

Sharma et al. [22] demonstrated that phages can play a role in scavenging biofilms by degrading the extracellular polysaccharides of *P. aeruginosa*. Indiani et al. [23] noticed, in in vitro experiments, that lysin CF-301 has an extremely strong ability to lyse biofilms and drug-resistant *S. aureus* biofilms that are formed in human synovial fluid. After adding CF-301, all biofilms in the catheter were removed, and the number of bacteria was reduced by five orders of magnitude. Poonacha et al. [24] found that lysin P128 could degrade host biofilms and kill bacteria within biofilms. Electron micrographs showed that 8.0 μg/mL of lysin P128 significantly degraded *Staphylococcus epidermidis*, *Staphylococcus Lugdunensis*, and *Staphylococcus haemolyticus* biofilms; 15.0–31.0 μg/mL of lysin P128 killed 99% of *S. epidermidis* and *S. haemolyticus* and 62.5 μg/mL killed 99% of *S. haemolyticus*. Singh et al. [25] determined the *Staphylococcus aureus* biofilm-disrupting ability of chimeric P1y187AN-KSH3b lysin and gentamicin via fluorescence imaging and demonstrated that the lysin exhibited an extremely strong *Staphylococcus aureus* biofilm-disrupting ability, whereas gentamicin alone failed to disintegrate *Staphylococcus aureus* biofilms. Lood et al. [26] observed the degradation effect of lysin P1yF307 on the biofilm of *Acinetobacter baumannii*; the *A. baumannii* biofilm growing on the catheter was significantly reduced after treatment with lysin P1yF307, both in vivo and in vitro. In addition, phage lytic enzyme LySMP was more than 80% effective in clearing biofilms formed by *Streptococcus suis* SS2-4 and SS2-H strains [27]; the removal rate of biofilms by phage or antibiotics alone was usually less than 20%, compared to that of the phage lytic enzyme LySMP. Furthermore, different cell wall endolysins encoded by *Streptococcus pneumoniae* or its phage could effectively eliminate the organism in vitro and in animal models [28]. Among them, LytA is a major *S. pneumoniae* autolysis enzyme, which is an N-acetyl cytosolic acyl-L-alanine amidase that removes biofilms formed by *S. pneumoniae*. In addition to LytA, other cell wall endolysins include LytC, Pal, Cpl-1, Cpl-7, and Ejl; furthermore, Lyt-A and Cpl-1 can act synergistically to effectively remove *S. pneumoniae* biofilms. These studies showed that phage-encoded endolysins exhibited better scavenging and killing effects on biofilms and the bacteria protected within them. This finding can be further exploited for future clinical management of infectious diseases associated with biofilms.

4 Improving the Anti-biofilm Activity of Phage Endolysins

The inhibitory effect of endolysin on biofilms has been previously studied, and the rate of endolysin inhibition in biofilms has been improved. Leitch et al. [29] investigated the ability of lactoferrin to enhance the activity of vancomycin and endolysin against the biofilms of a clinical *S. epidermidis* isolate. Lactoferrin treatment significantly ($p < 0.05$) reduced the number of viable biofilms and biofilm-released cells at an endolysin concentration of 16 mg/mL. The in vitro effect of endolysin (0–1000 μg/mL) on eukaryotic *Candida albicans* biofilm development was also investigated [30]. The action of lactoferrin is likened to that of cationic substances [31] such as protamine sulphate, which potentiates the action of vancomycin against *S. epidermidis* biofilms in vitro [32] and in vivo [33] and platelet microbicidal protein, which increases the vancomycin susceptibility of suspended S. aureus isolates [34]. In 2017, Hukić et al. [30] investigated two basic questions regarding endolysin activities on the selected microorganisms were investigated: (1) Whether endolysin inhibits biofilm formation and (2) Which concentration of the enzyme is required to change the natural biofilm-producing capacity of different strains of *S. aureus* (methicillin-sensitive and resistant), *S. pyogenes*, *P. aeruginosa*, and *Gardnerella vaginalis.* The effect of endolysin on the biofilm-forming capacity of 16 selected microbial strains was investigated in vitro using a test tube method

including four replicates; it was concluded that the potential of endolysin to alter biofilm-forming capacity depends on its concentration, the bacterial species, and the microbial strain used [30]. Some of the studies involving phage lysin treatment in bacterial biofilms and their characteristics are listed in Table 1.

4.1 Genetic Engineering Modification

4.1.1 Genetically Engineered Phage Endolysin Clears Biofilms

Many in vivo and ex vivo experiments have revealed the great potential of endolysins as an antibacterial agent [10, 45]. However, the lytic activity of endolysins against Gram-positive bacteria is higher than that of their Gram-negative counterparts. In addition, the host specificity and tendency to form inclusion bodies when expressed in prokaryotes limit the activity and application of endolysins for multiple bacterial infections (e.g., some mucosal infections). In addition, the C-terminal binding domain of endolysins has a strong hydrophobic and repetitive transmembrane region, which makes endolysins poorly water-soluble and reduces their application. To overcome these drawbacks and improve the lytic activity and host spectrum of endolysins, scientists have utilized molecular biology to design and modify natural phage endolysins to produce improved antibacterial reagents. Researchers have targeted the specificity of the C-terminal binding structural domain of phage endolysins to develop rapid detection reagents for bacteria. Bacterial biofilms exhibit resistance to antimicrobial therapy and clearance by the host immune system, making eradication very difficult. To address this issue, Lu et al. [47] engineered bacteriophage to express a biofilm-degrading enzyme during infection to simultaneously attack the bacterial cells in the biofilm and the biofilm matrix. The authors show that the efficacy of biofilm removal by this two-pronged enzymatic bacteriophage strategy is significantly greater than that of nonenzymatic bacteriophage treatment and cleared up 99.997% of the E. coli population in biofilms [46]. Therefore, the use of endolysins for treating bacterial biofilms has been supported and advocated by many researchers internationally.

4.1.2 Chimeric Enzymes

Some endolysins can alter their specificity and catalytic activity by substituting their structural domains. The peptide chain endonuclease structural domain of streptococcal phage lysin λSA2 (the λSA2 cleavage site is present on the peptidoglycan of *Streptococcus* and *Staphylococcus* [47]) was combined with the SH3b-binding structural domains of staphylococcal phage lysin LysK and staphylococcal endolysin to form a chimeric enzyme that not only produced higher lytic activity against *S. aureus* (including penicillin-resistant strains) but also maintained its original activity [48, 49]. Furthermore, the three fusion proteins HydH-5SH3b (HydH5 + SH3b structural domain), CHAPSH3b (cysteine and histidine-dependent aminohydrolase/peptidase (CHAP) structural domain + SH3b structural domain), and HydH5Lyso (HydH5 + Lysostaphin) of the *S. aureus* phagocytic lysin HydH5 and staphylococcal endolysin lysostaphin both exhibited higher cleavage capacity than HydH5 [50]. Jagielska et al. [51] combined LytM, an autolysis enzyme of *S. aureus*, with the CBD of the *S. aureus* lytic enzyme to create a chimeric enzyme with a lytic capacity that was 540-fold higher than that of the original lytic enzyme. Yang et al. [52] combined the CD of the lytic enzyme Ply187 from *S. aureus* and the binding domain of the phage lytic enzyme phiNM3 to form the chimeric enzyme ClyH. The latter not only increased the lytic capacity but also expanded the host spectrum. Dong et al. [53] combined the CD Ply187 N (1–157 aa) of the lytic enzyme Ply187 from *S. aureus* and the binding domain (146–314 aa, V12C) of the phage lytic enzyme PlyV12 to form the chimeric enzyme Ply187 N-V12C. This lysed not only *S. aureus* but also streptococci (*S. aureus*, *S. lactis*, *S. pyogenes*) and Enterococcus (*Enterococcus faecalis*), increasing the host spectrum of the lytic enzyme. The bactericidal function of endolysins was enhanced by combining different lytic enzyme structural domains. Yang et al. [54] constructed the lytic enzyme ClyH by fusing Ply187 (Pc) with the non-SH3b-like cell wall-binding structural domain of phiNM3, which exhibited good bactericidal activity in vitro and in vivo and disrupted the biological periplasm formed at different times [55]. Fernandes et al. [56] combined the CD of *E. faecalis* phage lysin F168/08 and the binding domain of phage87 lysin Lys87b to form a chimeric enzyme. The latter enzyme not only expanded the lysis spectrum but also increased its water solubility. Other investigators designed ClyS as a chimeric lysin by splicing the CD of phage Twort lysin plyTW and CBD of phage phNM3 lysin [57]. The designed C1yS lysin enhanced its water solubility and lytic activity, laying the foundation for clinical applications [58]. SINGH et al. [59] determined the biofilm-disrupting ability of chimeric lysins Ply187AN-KSH3b and gentamicin by fluorescence imaging method, respectively. The results demonstrated that the chimeric lysin Ply187AN-KSH3b had a strong ability to disrupt biofilms, while gentamicin could not lyse biofilms. The fusion protein SMAP-29-KZ144 was formed by fusing the antimicrobial peptide SMAP-29, which can penetrate the outer membrane of bacteria with the N-terminal end of lytic enzyme KZ144. The in vivo killing effect of a series of antimicrobial peptide endolysins designed along these lines against *P. aeruginosa* and its biofilms has been demonstrated in a nematode infection model [60].

Table 1 List of studies involving phage lysins processing in bacterial biofilms and their characteristics

Authors	Journal	Country(s)	Biofilm strains	Endolysins	Recombinant	Combination with other antimicrobial agents	Important conclusions
Kuiper Jesse et al. [35]	Biofouling	The Netherlands	*Staphylococcus aureus* (MRSA)	XZ.700 [35]	Chimeric endolysin, consisting of portions of the Staphylococcus aureus phage endolysin Ply2638 Abaev et al. (2013) and lysotaphin Sabala et al. (2014)	Cellular debris composition	The efficacy of endolysin XZ.700 (12.5–50 µg/mL) on MRSA biofilms on titanium at 24 and 48 h. The reduction of MRSA biofilms was 80–90%. PVP-I (0.35%) and gentamicin (1000 µg/mL) reduced the biofilms by 90–95%, and after 48 h the biofilms showed a stronger treatment resistance, and no such increase in resistance was observed with XZ.700 Biofilms with XZ.700 had similar reductions compared to the addition of DNaseI and the combination treatment method After exposure to the supernatant of XZ.700-treated 24 h biofilms and 48 h biofilms, compared to untreated controls, the amount of DNA exhibited similar, normal cell metabolism (between 79 and 112% for all results compared to control), and exposure to PVP-I (0.35%) and gentamicin (1000 mg/mL) derived lysates resulted in a significant reduction in DNA amount (37–69%)
Żebrowska Joanna et al. [36]	International Journal of Molecular Sciences	Poland	*Geobacillus stearothermophilus*	TP84_28 [36]	Introduce His-tag at the N-terminus	/	Treatment of bacteria with 0.2 µg TP84_28 reduced biofilm formation by 62.5%, while the addition of 2 µg TP84_28 reduced biofilm production by 94.4% and the addition of 20 µg TP84_28 reduced it by 99.2% Endolysin TP84_28 removed biofilm of *Staphylococcus aureus* by 85.9%, Streptococcus pyogenes by 62.5%, 53.2% for *Enterobacter cloacae* and 41.7% for *Streptococcus aureus*
Landlinger Christine et al. [37]	Antimicrobial agents and chemotherapy	Austria, Belgium	*Gardnerella*	PM-477 [37]	Introduce His-tag at the N-terminus	/	*Gardnerella* biofilms were tolerant to clindamycin (CLI) with a minimum biofilm eradication concentration of 512 mg/ml, metronidazole (MDZ) concentrations of 8–128 mg/ml removed biofilms above the lower limit of detection, and the minimum biofilm eradication concentration of endolysin PM77 (2–32 mg/mL) was lower than that of any antibiotic

Table 1 (continued)

Authors	Journal	Country(s)	Biofilm strains	Endolysins	Recombinant	Combination with other antimicrobial agents	Important conclusions
Johnston William et al. [38]	Biofilm	United Kingdom	*Gardnerella vaginalis*, *Fannyhessea vaginae*, *Prevotella bivia*, and *Mobiluncus curtisii*	CCB7.1 [38]	Introduce His-tag at the N-terminus	/	After treating the multispecies bacterial *vaginosis* model with 128, 256, and 512 μg/ml of endolysin CCB7.1, respectively, the viability of *Trichomonas vaginalis* in this model was significantly reduced by 1-2log10 compared to the untreated control. CCB7.1 was able to reduce the viability of *Trichomonas vaginalis*
Mikhail et al. [39]	Viruses	Russia, Germany	*Klebsiella pneumoniae* Ts 141–14	LysECD7 (*E. coli* phage) [39]	Introduce His-tag at the N-terminus	/	LysECD7 at concentrations of 1000 and 3000 μg/mL (62 and 186 μM) prevented biofilm formation by 74 and 79%, respectively, compared to the control group. Also, amikacin at concentrations of 50, 100, and 250 μg/mL (64, 128, and 320 μM) reduced biofilm density. Thus, in a close concentration range, the same effect was seen for both LysCD7 and amikacin At concentrations of 1000 and 3000 μg/mL (62 and 186 μM), the disruptive activity of LysECD7 against mature bacterial biofilms was higher than that of amikacin at 50–250 μg/mL (64–320 μM): bacterial biofilm density was reduced by 60 and 68% compared to the control group, 37%, 50%, and 49%, respectively LysECD7 can counteract emerging and forming biofilms in vitro and in vivo, with comparable activity to amikacin, but the inflammatory infiltration of peritoneal tissue and exudate accumulation in the area around the implant is less risky

Table 1 (continued)

Authors	Journal	Country(s)	Biofilm strains	Endolysins	Recombinant	Combination with other antimicrobial agents	Important conclusions
Ning Hou-Qi et al. [40]	International Journal of Food Microbiology	PR China	*Vibrio parahaemolyticus* (*V. parahaemolyticus*)	Lysqdvp001 [40]	Introduce His-tag at the N-terminus	Coupling with ε-polylysine	The concave lines connecting the bacterial growth inhibition zones on the microanalysis plates are consistent with the characteristics of the theoretical synergistic effect lines in tessellation analysis. In addition, the FIC (fractional inhibitory concentrations) of *Vibrio parahaemolyticus* calculated by the combination of 60 U/mL Lysqdvp001 and 0.2 mg/mL ε-PL was below 0.45. The antibacterial synergy between Lysqdvp001 and ε-PL was confirmed by checkerboard and turbidity. The antimicrobial synergy between Lysqdvp001 and ε-PL was confirmed by checkerboard and turbidity reduction assays
Baliga et al. [41]	Probiotics and Antimicrobial Proteins	India	*Aeromonas hydrophila*	LysE [41]	Introduce His-tag at the N-terminus	Combination with EDTA or mucilage	Endolysin has the highest wall lysis activity at pH 4 and maintains more than 50% activity at pH 10. The endolysin showed more than 50% activity even after 30 min incubation at 100 °C. Also, endolysin LysE resulted in one log reduction in CFU/mL in 30 min and showed anti-biofilm ability in combination with EDTA. Checkerboard analysis showed synergistic effects against *Aeromonas hydrophila* in combination with lower concentrations of mucilage. LysE can be used with outer membrane permeabilizers to expand the arsenal against Gram-negative bacteria in the aquaculture, food, and medical industries
Oh et al. [42]	Viruses	Korea	*Enterococcus faecalis*	Lysin from phage PBEF129 [42]	Introduce His-tag at the N-terminus	Combination with cefotaxime	Combined treatment with cefotaxime and endolysin (CFT + E) showed synergistic effects. Cefotaxime or phage endolysin removed bacteria to a limited extent, while the application of phage PBEF129 to biofilms resulted in a significant reduction in bacterial load

Table 1 (continued)

Authors	Journal	Country(s)	Biofilm strains	Endolysins	Recombinant	Combination with other antimicrobial agents	Important conclusions
Oliveria et al. [43]	PLoS ONE	Portugal, India, Belgium	A wide panel of Gram-negative bacteria (13 different species)	Lys68 [43]	Introduce His-tag at the N-terminus	Combination with malic or citric acid	Lys68 is able to lyse a wide panel of Gram-negative bacteria (13 different species) in combination with the outer membrane permeabilizers EDTA, citric and malic acid. While the EDTA/Lys68 combination only inactivated *Pseudomonas* strains, the use of citric or malic acid broadened Lys68's antibacterial effect on other Gram-negative pathogens (lytic activity against 9 and 11 species, respectively). Particularly against *Salmonella Typhimurium* LT2, the combinatory effect of malic or citric acid with Lys68 led to approximately 3 to 5 log reductions in bacterial load/CFUs after 2 h, respectively, and was also able to reduce stationary-phase cells and bacterial biofilms by approximately 1 log
Jie Zhang et al. [44]	Clinical Laboratory	PR China	*Acinetobacter baumannii* and Biofilm-Bound *A-baumannii*	LysAB3 [44]	Introduce His-tag at the N-terminus	Combination with 3 types of antibiotics	After 24-h treatment with either bacteriophage AB3 and its LysAB3, *A. baumannii* biofilms were significantly degraded, and the number of viable biofilm-bound *A. baumannii* was also significantly decreased. After removing the amphiphilic peptide structure motif from LysAB3, the antibacterial activity decreased from 95.8 to 33.3%. LysAB3 can effectively degrade *A. baumannii* biofilm and biofilm-bound *A. baumannii* in vitro

4.1.3 Endolysin Truncation

Notably, some endolysins, such as Mur encoded by *Lactobacillus deuterium* phage LL-H, still induced lytic activity when the C-terminus was removed, whereas other enzymes had increased activity with the deletion or partial deletion of the C-terminus [61]. Loessner et al. [62] noticed that the whole enzyme activity of *S. aureus* lytic enzyme P1y187 was low, but its N-terminal amino acid (1–157 aa) had high activity, whereas 158–227 aa and 158–628 aa were inactive. Mutant forms of group B streptococcal lysin P1yGBS with multiple fragment losses exhibited increased activity, retaining only the N-terminal 1–141 aa and C-terminal 13 amino acids, with a 28-fold higher lytic activity than the full enzyme [62]. Meng Wu et al. [63] found that when the whole enzyme of Ply187 was cut off and only its CHAP structural domain was expressed, it showed strong antibacterial activity against both S. aureus and its biofilms. When some skin chains of the staphylococcal lytic enzyme LysK were truncated, leaving only the CHAP structural domain, it maintained its staphylococci (including MRSA) lysing activity [64, 65]. The *Clostridium difficile* phage lysin CD27L was truncated, leaving the N-terminal domain $CD27L_{1–179}$, which not only increased the lytic activity against *C. difficile* but also expanded its lytic spectrum, whereas the other half of the lysin, $CD27L_{180–270}$, had no lytic activity [66]. Thus, this is another way to modulate the specificity of the lytic enzyme. A truncated lytic enzyme, even a single structural domain protein with a greatly reduced relative molecular mass, might reduce the mounting of an immune response. Fenton et al. [67] also demonstrated that the peptidase CHAPk, produced by the truncated structural domain (cysteine, histidine-dependent amidohydrolase/peptidase) CHAPK from phage K lysin LysK of *S. aureus*, can act as a biocide to rapidly degrade the biofilm formed by *S. aureus* and prevent and treat biofilm-associated staphylococcal infections. Pure CHAPk can eliminate biofilms of *S. aureus* DPC5246 within 4 h. In addition, CHAPk prevented the formation of *S. aureus* DPC5246 biofilms and reduced the number of *S. aureus* colonies on the skin surface.

By modifying the endolysins, we can increase their lytic activity and make them more target specific for different pathogenic bacteria to optimize the endolysins. In conclusion, combining different structural protein domains allows the design of endolysins with high activity against bacterial biofilms, laying the foundation for future clinical applications.

4.2 Combining Enzymes with Antibiotics or Membrane Permeation Agents

Several endolysins combined with antibiotics can result in synergistic effects and improve bactericidal effects. Mixing the endolysins HydH5 and LysH5 of *S. aureus* phage phiIPLA88 with different sites of action produced better in vitro anti-staphylococcal effects [48]. Notably, the combined use of the lytic enzyme Cpl-1 (2.5 μg) and Pal was more bactericidal than when 5 μg of Cpl-1 or Pal was used alone [68]. Other studies also confirmed that the combination of the lytic enzyme LysK and staphylococcal lysins exhibited synergistic effects [69]. In addition, the combination of *S. pneumoniae* lytic enzyme Cpl-1 with antibiotics has similar synergistic effects. Cpl-1 combined with gentamicin at less than the minimal inhibitory concentration can improve the killing of *S. pneumoniae*. Cpl-1 can also synergize penicillin to lyse penicillin-resistant bacteria. Therefore, a rational combination of enzymes and antibiotics can potentially control specific antibiotic-resistant bacteria [70]. McCarthy noted that Exebacase (Lysin CF-301) is an attractive antimicrobial agent because it demonstrates rapid bacteriolytic activity against staphylococcal species, including Staphylococcus aureus, has a low resistance profile, eradicates biofilms, and acts synergistically with other antibiotics [71].

In recent years, phage endolysins have also been studied in combination with membrane permeabilizers [e.g., polymyxin B and ethylene diamine tetraacetic acid (EDTA)] to overcome the outer membrane barrier of Gram-negative bacteria. Briers et al. [72] combined *P. aeruginosa* phage lysin OBPgp279 and *Salmonella* phage PVP-SE1 gp146 with various outer membrane permeating agents (e.g., polycationic peptide, hydrophobic pentapeptide, parasin l, and lycotoxin l) to form fusion proteins. "Artilysins" (outer membrane-penetrating endolysins) exhibit superior lytic activities in vitro. The phage lytic enzyme SPN1S [73], which has an endolysin-like superfamily domain, can kill most Gram-negative strains and maintain stable antibacterial activity at different pH (pH 7.0–10.5) and temperature (25–45 °C) ranges. When combined with the chelating agent EDTA, the ability of SPNlS to pass through the bacterial outer membrane and its lytic activity was significantly enhanced. Another lytic phage Ts2631 reduced all Enterobacteriaceae pathogens, including multi-drug-resistant *Citrobacter*, below the detection limit [by 6(log (CFU/mL))] [74] when combined with EDTA. Liu et al. [75] identified and prepared two phage endolysins, LysWL59 and LysWL60, from phage LPST10. The lytic activity of both enzymes was extensive against Gram-negative bacteria after chloroform treatment. LysWL59 showed more stability than LysWL60 and maintained good lytic activity at pH 6.0–10.0 and a temperature of 4–90 °C. When LysWL59 was combined with an outer membrane-permeant, live *Salmonella typhimurium* cells suspended in Tris–HCl buffer were lysed. LysWL59 (2.50 mmol/L) in combination with EDTA (0.50 mmol/L) removed 93.03% of *S. typhimurium* biofilms on lettuce within 1 h [75].

4.3 Binding to Drug Molecules

Numerous recent studies have combined endolysins with materials such as drug molecules to improve the stability and antimicrobial properties of endolysins. The potential of using nanoparticles (NPs) for biofilm control and eradication has attracted increasing scientific interest [76, 77]. In 2017, Liu et al. immobilized endolysin proteins on a layered zeolite imidazole acid framework (ZIF-8) and analyzed the interaction between $AgTiO_2$ nanoparticles and endolysin, providing important biological applications [78]. Wang et al. [79] obtained endolysin-immobilized chitosan nanoparticles (Lys-CS-NPs) by integrating endolysin into chitosan nanoparticles (CS-NPs) by using an ionic gelation technique, which significantly improved the thermal stability and reusability of endolysin. Furthermore, Lys-CS-NPs exhibited excellent bacterial inhibition based on in vitro killing kinetics and the minimum inhibitory concentration of CS-NPs and Lys-CS-NPs against *P. aeruginosa* [79]. Liu et al. [80] showed that coating endolysin with polyγ-glutamic acid and chitosan could broaden the antibacterial spectrum and improve the antibacterial activity of composite endolysin nano-reagents. Chhibber et al. used divalent cobalt ions on plates to limit iron content and combined with phage KP01K2, NDP, and endolysin and found that the formation of biofilm of Klebsiella pneumoniae B5055 was prevented. From this result, it is clear that the combination of iron antagonists such as $CoSO_4$ and phage endolysin can be used as an adjuvant therapy to prevent bacterial biofilm formation [81]. Zhang et al. [82] showed by laser confocal microscopy supplemented with electron microscopy that combined treatment with phage, endolysin, and chlorine was an effective method for controlling and eliminating bacterial biofilms on various surfaces. The combined use of 3×10^7 PFU/mL of phage and 210 mg/L of chlorine was able to reduce the growth of 94% of the biofilm and remove 88% of the formed P. aeruginosa biofilm.

5 Conclusion

Bacterial phage endolysins can break the cell wall of bacteria rapidly and efficiently, and no direct adverse effects on humans have been reported. Its specificity is between that of antibiotics and phages, and bacteria are less likely to develop tolerance to it. Currently, it is possible to transform bacteria using recombinant DNA and plasmids, thus expressing the target lytic enzyme in large quantities, making it easily available. Compared with conventional antibiotics, endolysins also possess the following unique properties. (1) An evolutionary advantage: endolysins are derived from phages that have co-evolved with their host bacteria. This phenomenon has been preserved by natural selection. (2) High specificity: the functional endolysin domain that binds to the bacterial cell wall recognizes only specific species of bacteria, making endolysins highly specific. (3) High bactericidal activity: endolysins are "weapons" for releasing daughter phages and are thus naturally efficient. (4) Very low potential for drug resistance development: owing to the pressure of natural selection, phage endolysins only act on the essential and conserved parts of the host bacterium, and bacteria are rarely able to develop resistance to escape this recognition. Therefore, it has some advantages as a novel antibacterial drug. However, phage endolysins have also some problems: (i) some natural endolysins expressed in E. coli are toxic to the expressing strain, and proteins are often expressed in inclusion bodies [83], (ii) endolysins are vulnerable to protease attack after entering the organism and have a short half-life [84], and (iii) it is difficult to determine the optimal time and optimal dose of endolysins in the treatment process [84]. Although endolysins have some drawbacks, theoretical and experimental studies support the use of genetic engineering and protein engineering to mutate prophage genes, replace lytic enzyme genes, modify structural domains, synthesize lytic enzyme chimeras with different lytic activities, and modify and optimize endolysins to achieve the goals of high yield, high efficiency, broad spectrum, and stability, making them ideal antimicrobial substances that clinicians seek. Endolysin has a large developmental value in antibacterial activity and is expected to be an effective candidate for solving the problem of drug-resistant bacteria through continuous research. Thus, phage endolysins may be indispensable weapons against pathogenic biofilms.

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Declarations

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