Chapter 11

Physiology and Pathology of Multidrug-Resistant Bacteria: Phage-Related Therapy

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.70487

Abstract

Multidrug-resistant bacteria (MDR) are spreading rapidly across the world that outpace development of new antibiotics. Options other than antibiotics treatment are urgently needed. In this chapter, we review the current status of nonantibiotics-based strategies including phage therapy and phage-derived protein therapy for targeting Gram-positive strains (methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium*) and MDR Gram-negative strains (*Acinetobacter baumannii* and *Pseudomonas aeruginosa*).

Keywords: multidrug-resistant bacteria, MDR, MRSA, VRE, *A. baumannii*, *P. aeruginosa*, infection, biologics, bacteriophage, lysin, endolysin, phage therapy

1. Introduction

Host-pathogen battle is a never ending theme regarding infection and immunity. Human innate immune defense is triggered at early stages of bacterial infections. As the major players of innate immunity, macrophages, neutrophils, dendritic cells (DCs) and natural killer cells recognize pathogen-associated molecular patterns (PAMPs) and damage (or danger)-associated molecular patterns (DAMPs) through their pattern recognition receptors (PRRs) [1–3].

Known PRRs consist of Toll-like receptors, C-type lectin receptors, Nod-like receptors (targeting intracellular pathogens via inflammasome), AIM2-like receptors, RIG-I-like receptors and microbial nucleic acid sensors [1, 4, 5]. Identified PAMPs include lipopolysaccharide (LPS or endotoxin), peptidoglycan, lipoteichoic acid, exotoxin, effector protein, lipoprotein, porin, flagellin, pilin, glycoprotein, glycosylphosphatidylinositol, microbial nucleic acid and outer

membrane vesicle (extracellular vesicle or exosome) [1, 4, 6, 7]. Activation of innate immune systems through interactions of PAMPs and DAMPs with PRRs induces antigen presenting cells (APCs, mainly macrophages and DCs) to phagocytose bacterial pathogens and cleave pathogen-related proteins to peptides within endosomes and lysosomes [8]. The cleaved peptides can be recognized by major histocompatibility complex (MHC)-II and presented to the surface of APCs. MHC-II-peptide complex is the natural ligand of T-cell receptor (TCR) from $CD4^+$ T cell that can stimulate cytokine and chemokine secretion, inflammatory signaling cascade and activate adaptive immune responses from both T lymphocytes and B lymphocytes for protective immunity and elimination of pathogens [1, 4, 8].

On the other hand, bacteria evolve strategies to compromise, manipulate or evade host immune system that can lead to host cell autophagy and pyroptosis and thus enhance bacteria adhesion, colonization and chances of survival within the host [9, 10]. Moreover, excessive or chronic inflammations induced by bacterial infections are closely related with pathogenesis of autoimmune disorders [11]. Thus, effective treatment of bacterial infections is urgent. However, multidrug-resistant bacteria (MDR) appear to outpace current development of new antibiotics, especially to six frequently reported MDR bacteria, designated as *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp. (ESKAPE) [12, 13].

Staphylococcus aureus (*S. aureus*) is a Gram-positive, leading nosocomial pathogen that can cause many types of infections, ranging from surgical site infections from intensive care units (ICUs) to community-acquired skin and soft tissue infections. Methicillin-resistant *S. aureus* (MRSA) became endemic in hospitals by the 1980s and in some areas, more than 50% of *S. aureus* isolates are now resistant to methicillin [14]. In the United States, an estimated 80,000 invasive MRSA infections and 11,000 related deaths occur annually [15].

Enterococci are Gram-positive, facultatively anaerobic cocci that often occur in chains of various lengths. *Enterococci* are generally considered as low virulent as evidenced by their natural presence in human gastrointestinal tract and long being used as probiotics in human. They have attracted more attention since increasing number of patients who are immunosuppressed or receiving antimicrobial agents have been reported to suffer from MDR *Enterococci* infections [16]. In fact, an estimated number of 20,000 cases and 1300 deaths are caused by vancomycin-resistant *E. faecium* (VRE) infection annually in United States [17].

Pseudomonas aeruginosa (*P. aeruginosa*) is a Gram-negative, leading cause of nosocomial infections and shows potential of rapid evolution of antibiotics resistance during therapy [18]. Susceptible individuals include victims of cystic fibrosis and those with an impaired immune system caused by HIV infection, organ transplantation, cytotoxic drugs or burns with vascular damage [19].

Acinetobacter baumannii (*A. baumannii*) is a Gram-negative, drying and disinfectant-resistant bacterium that can evade human immunity and develop drug resistance to almost all classes of antibiotics [20, 21]. MDR *A. baumannii* infection is mainly restricted within hospitals for patients with mechanical ventilation, burns, wounds, sepsis, meningitis and often leads to high morbidity and mortality [20, 22, 23].

http://dx.doi.org/10.5772/intechopen.70487

Bacteriophage (short for phage), as its name indicates, is a natural virus that only infects bacteria and this unique property makes bacteriophage an attractive alternative for bacterial infection treatment, especially for the current MDR bacteria spreading worldwide. This chapter reviews the current status of phage therapy and phage-derived protein therapy for Gram-positive strains including MRSA and VRE and MDR Gram-negative strains (*A. baumannii* and *P. aeruginosa*).

2. Lytic bacteriophage structure

Phages are estimated to be the most diverse and abundant entity on earth that exist in every ecosystem with the range of 10^{30}–10^{31} and are about 10 times more than their bacterial hosts [24]. For instance, agricultural soils usually harbor a phage count of approximately 10^{8}–10^{9} per gram of dry soil and aquatic environments contain a phage titer of 10^{4}–10^{8}/mL [25–28].

The basic phage structure is made up of a hexagonal head, which harbors the phage double-stranded DNA (dsDNA), together known as capsid, a tail and a connector between head and tail (**Figure 1A**) [29]. The head is anchored to a tail sheath via a neck and a collar and ends into a hexagonal base plate. Tail fibers/spikes emerge from the base plate and the tail sheath tapers into a tail tube.

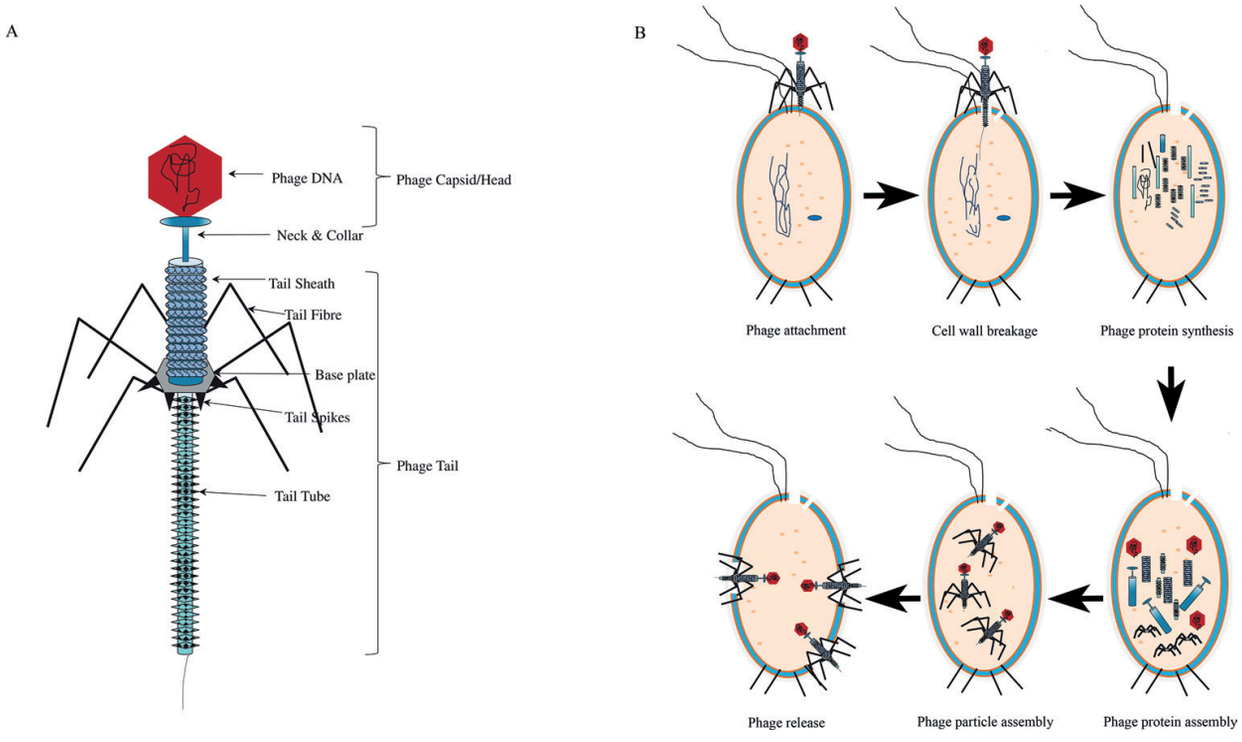

Figure 1. Structure and life cycle of a lytic bacteriophage. (A) The structure of a typical lytic bacteriophage is shown. (B) Lytic phage life cycle is shown starting with attachment on bacterial cell surface and proceeding to phage release by intermediate steps.

Phages are classified into two categories (lytic and nonlytic or temperate) and 13 families based on certain criteria including its host specificity, morphology, genotype, infective mode, with or without envelope and lipid [30]. Currently, over 5500 different bacteriophages have been sequenced and 96% of them, including most of therapeutic phages, belong to the order *Caudovirales* [31]. The order *Caudovirales* comprises three families according to the morphological features of the tail: *Myoviridae* (with long, rigid, contractile tails, e.g. T4), *Siphoviridae* (with long, flexible, noncontractile tails) and *Podoviridae* (with short, noncontractile tails).

3. Lytic bacteriophage life cycle

Lytic bacteriophages are of special interest in phage therapy of bacterial infections. Lytic phage life cycle typically consists of (1) Attachment/adsorption to the host cell—it involves the contact between tail fibers and the host cell receptors like lipopolysaccharide (LPS), peptidoglycan (PG), outer membrane (OM), fimbriae, flagellum or sex pilus; (2) Injecting phage DNA—the phage secretes specialized enzymes that destroy the LPS, PG and OM to inject the phage DNA through the tail tube into the host cell; (3) Phage DNA replication—after phage DNA injection, phage early genes are expressed which take the control of host cell machinery to replicate phage DNA. The replicated phage DNA then expresses phage late proteins necessary for virion assembly; (4) Assembly and packing of phage particle—once the assembly proteins are expressed, capsid assembles by encapsulating the phage genetic material and later a separately assembled tail joins the capsid to make a full phage particle; and (5) Host cell lysis and phage release—the phage late proteins comprise endolysins and holins which together break the PG layer, lyse the bacteria and burst out the fully formed bacteriophage into the environment [32, 33]. **Figure 1B** illustrates a cartoon process of how bacteriophage infects, lyses bacteria and releases progeny. Since the lytic phage kills the bacterial host cell after completing the lytic life cycle, they are seen as potential antibacterial agents.

4. Phage therapy against MRSA

More than 200 lytic phages against *S. aureus* have been characterized [31]. Most of *S. aureus* phages belong to the *Siphoviridae*, such as lytic phage ϕMR11 [34] and lytic phage phiIPLA35 [35]. A small number of *S. aureus* phages belong to the *Podoviridae* like SAP-2 phage [36] and *Myoviridae* like Stau2 [37] and well-known phage K [38].

Development of phage resistance to host-pathogen and cross-resistance with antibiotics are seldomly observed [39]. Thus, MRSA pathogens can be targeted by the anti-*S. aureus* phages such as phage K and φMR11 [34, 40]. φMR11, administrated intraperitoneally, appeared rapidly in the circulation of mice challenged with fatal *S. aureus* infection and successfully protected mice without any adverse effects [34].

S. aureus-specific phage MR-10, when combined with Mupirocin, can not only significantly reduce the *in vitro* adherence, invasion and cytotoxicity of MRSA on murine nasal epithelial

http://dx.doi.org/10.5772/intechopen.70487

cells and effectively eradicate MRSA population from mouse nares but also decrease the frequency of mutation coupled with Mupirocin treatment alone to negligible levels [41]. Similarly, synergistic effect on anti-*S. aureus* was observed when combination of *S. aureus* phages with gentamicin or linezolid was used [42, 43].

Biofilms play a key pathological role in *S. aureus*-associated chronic infections [44]. Bacteriophage cocktail NOV012 containing two different phages, P68 and K710, showed high protection against MRSA-related chronic rhinosinusitis [44]. Moreover, Poland scientists demonstrated that efficient phage therapy was an alternative to antibiotics for treating chronic MRSA infections with significant savings in healthcare costs [45].

Interestingly, researchers found that some *S. aureus*-specific lytic phages, identified from natural sewage, showed higher protective efficiency against MRSA in mice than antibiotic or conventional phage and antibiotic combined treatment [46, 47].

To overcome the rapid release of toxics arising from lytic phage induced *S. aureus* lysis, the endolysin gene controlling the release of phage progeny was inactivated in *S. aureus* phages. These lysis-deficient phages successfully induced MRSA death in mice infection model without lysis induced side effects such as septic shock or toxic shock syndrome, possibly based on the sole activity of holin [48, 49].

Phage can be used as an efficient carrier to bring photosensitizers (light-activated antimicrobial agents) to *S. aureus* by chemical conjugation which then resulted in enhanced and selective killing of MRSA when exposed to low-dose red light [50]. Moreover, as the carrier for photosensitizers, the ability to selectively kill MRSA is independent of phage's ability to infect *S. aureus* [51].

5. Phage therapy against VRE

More than 27 phages have been isolated and tested for their protective efficacy VRE infection [52]. Most of these phages belong to the *Myoviridae* or the *Siphoviridae* families [52]. Phage ENB6, isolated from raw sewage, has lytic activity against a wide range of clinical VRE isolates and single dose of intraperitoneal injection was sufficient to rescue 100% of the fatally infected mice [53]. The authors also demonstrated that the ability of this phage to rescue bacteremic mice was not due to a nonspecific immune effect but due to the ability of phage ENB6 itself [53]. Similarly, *in vivo* therapeutic potential of virulent phage phiEF24C, evaluated in a sepsis BALB/c mouse model, proved to be effective against lethal VRE infection at a low concentration following a single or repeated phage exposure [54]. *Enterococcus faecalis* phage IME-EF1 was isolated from hospital sewage; when administrated intraperitoneally in a murine sepsis model, one dose of IME-EF1 or its endolysin was found to reduce the bacterial blood count and protect the mice from a lethal challenge of *E. faecalis* [55]. Biofilm-associated VRE infections are challenging for treatment. EFDG1, isolated from sewage water, was efficient not only in nearly eliminating 2-week old *E. faecalis* biofilms of around 100 μm thickness but also in prevention of *E. faecalis* root canal infection [52, 56].

6. Phage therapy against MDR *P. aeruginosa*

More than 110 phages specifically target *P. aeruginosa* and around 60% are lytic phages, which are frequently isolated from hospital wastewater and sewage wastewater [57]. Fu et al. [58] used an *in vitro* model to investigate the effect of lytic phages in the prevention of *P. aeruginosa* biofilm formation in hydrogel-coated catheters and found that catheters, when pretreated with single phage, presented high reduction of biofilm formation at early inoculation while cocktail phage treatment in keeping high reduction of biofilm formation lasted over 48 hours post treatment.

Torres-Barceló et al. [59], Knezevic et al. [60], Zhang and Hu [61] and Oechslin et al. [62] explored the combinatorial effect of phages with different antibiotics against *P. aeruginosa* and found that certain combination can lead to synergistic effect than single treatment alone. Moreover, Torres-Barceló et al. found that long-term combination of phages with antibiotics not only showed synergistic benefit but also weakened antibiotics-induced resistance for *P. aeruginosa* when used alone [63].

A mouse lung infection model was used to evaluate therapeutic and prophylactic efficiency of phage PAK-P1 against MDR *P. aeruginosa* by nasal. The curative treatment of one single dose 2 hours after bacterial infection allowed over 95% survival and preventive treatment with single dose 4 days before infection resulted in 100% survival whereas untreated mice all died within 2 days after infection [64].

To evaluate efficacy and safety of bacteriophage therapy in human, Wright et al. used a phage cocktail named as Biophage-PA to carry out the first controlled clinical trial phase I/II for treating MDR *P. aeruginosa* that caused chronic otitis in 2009 [65]. Encouragingly, Biophage-PA-treated patients showed significant clinical improvements and no related side effects or local systemic toxicities when compared with placebo control individuals [65].

7. Phage therapy against MDR *A. baumannii*

Phage AB1 and phageφAB2, as the early characterized phages in detail against MDR *A. baumannii*, were reported in 2010 [66, 67]. Phage AB1 belongs to the *Siphoviridae* family and harbors a narrow host range, a latent period of 18 minutes and a burst size of 409, whereas phage φAB2 is from the *Podoviridae* family, showing rapid adsorption (more than 99% absorbed in 6 minutes), a latent period of less than 10 minutes, a burst size of around 200 and a broad host range [66, 67]. Furthermore, phageφAB2 was shown to be used potentially as an anti-MDR *A. baumannii* hand wash [68]. Two *A. baumannii*-specific lytic phages, AB7-IBBI and AB7-IBB2, belonging to the *Siphoviridae* family and the *Podoviridae* family, respectively, demonstrated the ability to remove approximately 75% of preformed biofilms of MDR *A. baumannii* and showed potential application in hospital as environmental biocontrol agent [69, 70]. vB_AbaM-IME-AB2, a novel lytic *A. baumannii* phage, belongs to the *Myoviridae* family with a latent period of 20 minutes and a burst size of 62 and can infect MDR clinical isolates of *A. baumannii* [71].

Mouse infection model-based studies showed that BS46, a specific *A. baumannii* phage, could protect mice infected intraperitoneally with five times the lethal dosage 50 (LD50) of a highly virulent *A. baumannii* strain [72]; and a five-membered *A. baumannii*-specific phage cocktail demonstrated therapeutic efficacy against MDR *A. baumannii* pathogen in an infected wound model [73].

Eight lytic phages, isolated from hospital sewage, can lyse 34 clinical *A. baumannii* strains with various spectrums [74]. One phage named as φkm18p, belonging to the *Podoviridae* family, showed potent lysis of 15/34 clinical *A. baumannii* strains, of which many were "extensively drug resistant" *A. baumannii* strains [74]. The authors suggested that a cocktail of φkm18p with other lytic phages has potential to treat all MDR *A. baumannii* strains [74].

Recently, other lytic phages or phage cocktails have been reported to have potentials for treatment of *A. baumannii* infections in ICUs including vB_AbaM_Acibel004 and podovirus vB_AbaP_Acibel007 [75], phage Bφ-C62 [76], vB-GEC_Ab-M-G7 [77] and vB_AbaM-IME-AB2 [78]. Of note, cleaning of ICUs with addition of active phage aerosol significantly reduced *A. baumannii* infection rate and consumption of antimicrobials [79], highlighting the potential of phage-based prevention and therapy against MDR *A. baumannii* in the near future.

8. Bacteriophage derived proteins as antibacterial biologics

Bacteriophage encodes specialized proteins that mediate the phage entry into and exit out of the bacterial host during the lytic cycle. These phage proteins/enzymes are critical for both disintegration of the physical barrier and exploiting physiological pathways to establish an infection. The bacterial cell wall comprises an outer membrane exopolysaccharides (OM-EPS) and inner membrane peptidoglycan (IM-PG), which serves as the target of various phage enzymes. Therefore, phage enzymes are perceived as "natural antibiotics" but the idea itself has remained in its infancy due to the largely popular and broadly effective antibiotic drugs. However, a growing number of MDR bacterial pathogens have rung the alarm and triggered a renewed interest in employing phage-derived proteins to treat bacterial infections. In this section, we will focus on the phage lysins, which enzymatically cleave the linkages in the peptidoglycan (PG) layer of the bacterial cell wall. The PG layer is made up of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) units in which the MurNAc residues are covalently linked via amide bonds to the L-alanine of the stem peptide [80]. The PG layer provides the structural integrity and rigidity to bacterium and its breakdown is essential for phage to enter and exit its host cell (**Figure 2**). Based on their temporal expression, phage lysins can be divided into two types: (1) virion-associated peptidoglycan hydrolases (VAPGHs) and (2) endolysins. **Figure 3** shows overall architecture of VAPGHs and endolysins. We present an overview of phage lysin function and therapeutic potential in treating bacterial infections. We also present the protein engineering strategies employed to enhance bacteriolytic property and tropism of such proteins.

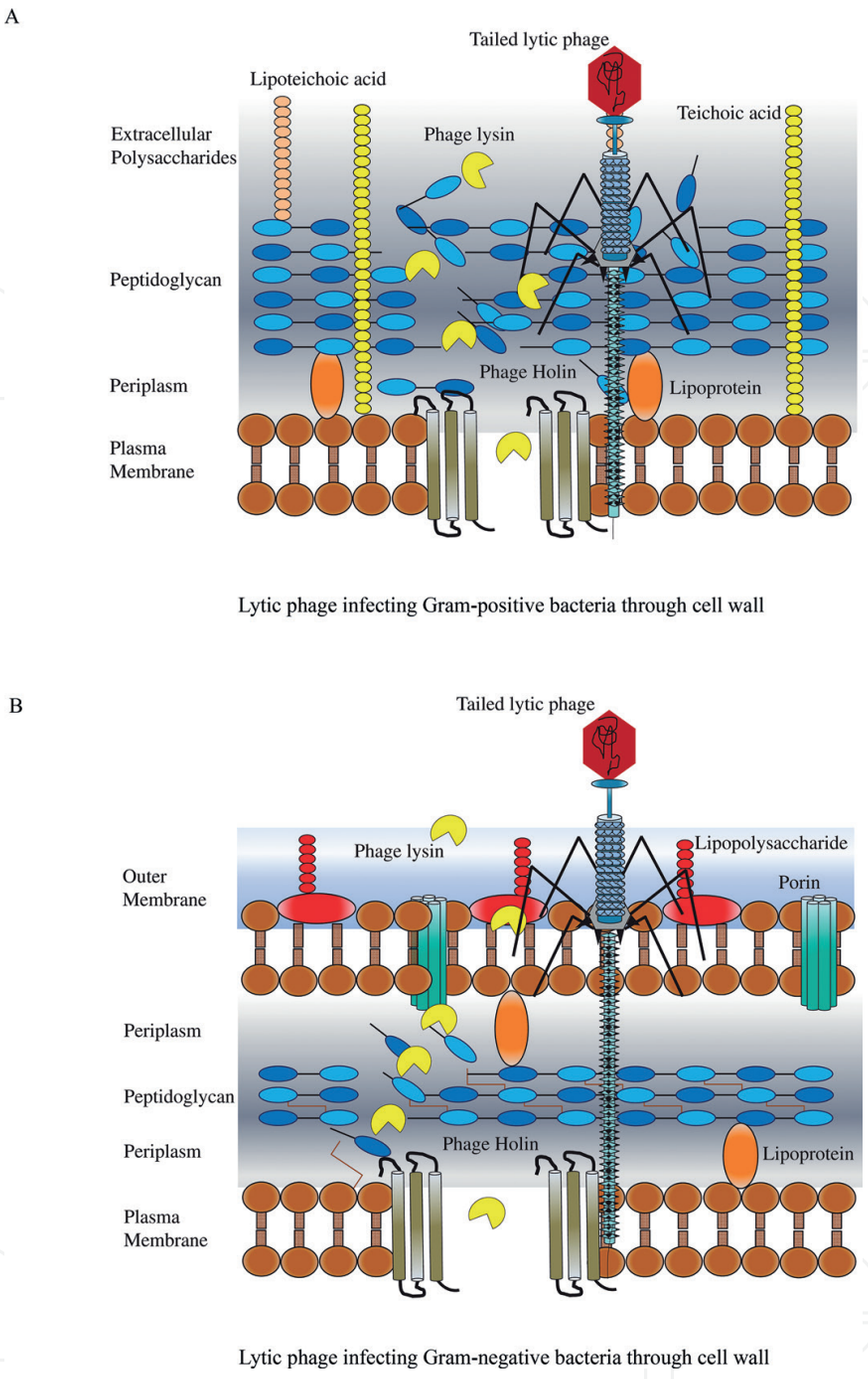

Figure 2. Bacterial cell wall degradation by phage enzymes. The bacteriophage enzymes comprising endolysin and holin together facilitate the degradation of the host bacterial cell-wall lysis by cleaving specific linkages in peptidoglycan layer and plasma membrane of (A) Gram-positive bacterial and (B) Gram-negative bacterial cell wall.

8.1. Virion-associated peptidoglycan hydrolases (VAPGHs)

VAPGHs are phage encoded hydrolytic enzymes which specifically degrade the PG layer of both Gram-positive and negative bacteria. VAPGHs are expressed early in the phage life cycle to degrade the OM for phage attachment and subsequent adsorption. These enzymes can have wide occurrence since the PG layer is common to both Gram-positive and Gram-negative bacteria.

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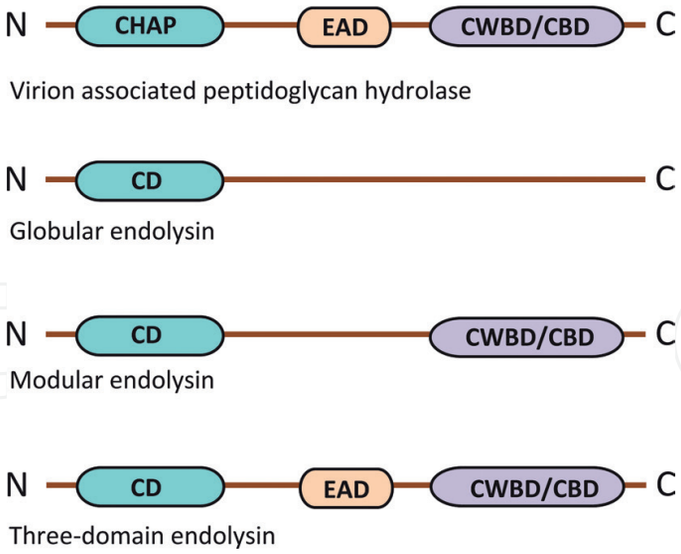

Figure 3. Domain architecture of phage muralytic enzymes. CHAP: cysteine/histidine-dependent amidohydrolases/peptidase; CD: catalytic domain; EAD: enzymatically active domain; CBD/CWBD: cell/cell wall binding domain.

However, it is thicker in Gram-positive organisms (20–80 nm) when compared to Gram-negative bacteria (10 nm) [80]. Different phage proteins can have various muralytic activities and their locations vary on the phage structure [81–83]. The VAPGH genes, although are not critical for phage multiplication, can ease out the phage infection process during suboptimal conditions [84, 85], against bacteria possessing an extensively cross-linked PG layer [83, 86] or if phage lysozyme activity is inhibited [87].

The muralytic activity of VAPGHs resides in the N-terminal and the cell wall/cell binding domain (CWBD/CBD) is present at the C-terminal (e.g. in a broad-spectrum staphylolytic phage P68 VAPGH P17) [88]. Owing to their modular architecture, VAPGHs can be engineered to enhance lytic activity and increase the tropism. A chimeric VAPGH P16-17 with N-terminal endopeptidase domain of Lys16 and the C-terminal CWBD of VAPGH P17 exhibits staphylolytic activity [89]. Chimeric versions of VAPGH HyDH5 and Lys16, produced by C-terminal fusion of bacteriocin lysostaphin SH3bCBD or a direct fusion of cysteine/histidine-dependent amidohydrolases/peptidase (CHAP) domain to SH3bCBD in the absence of enzymatically active domain (EAD), improve the lytic activity against *S. aureus* including MRSA, *S. epidermis and S. carnosus* [90–92]. This increased staphylolytic activity and tropism are attributed to the dual enzymatic activities targeting distinct linkages within the PG layer [90]. Similarly, *S. aureus*-specific temperate phage DW2 codes a hydrolytic VAPGH THDW2. This enzyme has a modular structure with N-terminal CHAP domain and an EAD at the C-terminal but lacks the CWBD/CBD [93].

8.2. Endolysins—phage enzymes degrading peptidoglycan

Endolysins are also muralytic enzymes like VAPGHs coded by dsDNA phage. Unlike VAPGHs, which act to degrade PG layer for phage DNA entry, endolysins are responsible for the release of the progeny phage late during the lytic phage cycle [94, 95]. Endolysins can be structurally divided into (1) globular endolysins which are constituted by a single catalytic domain (CD)

[83, 96]; (2) modular endolysins which are constituted by an N-terminal CD and a C-terminal CWBD/CBD [83, 97] and (3) three-domain endolysins which are constituted by CD, CWBD/CBD and an additional EAD in between CD and CBD [98]. The CD of different endolysins may have different enzymatic activities to cleave distinct linkages in the PG layer, whereas the CWBD is mainly responsible for imparting specificity of the interaction which can even be restricted to a particular serovar [99]. They can be further classified according to their functionality into (1) N-acetyl-β-D-muramidases with activity against MurNAc-GlcNAc linkages; (2) lytic transglycolases, which cleave N-acetylmuramoyl-β-1,4-N-acetylglucosamine bond; (3) N-acetyl-β-D-glucosaminidase cleaving the N-acetylglucosaminyl-β-1,4-N-acetylmuramine; (4) N-acetylmuramoyl-L-alanine amidases, which break amide bond between sugar and peptide and (5) endopeptidases, which cleave the peptide bond between two amino acid residues of the stem [94, 100].

The bactericidal property of the endolysins makes them attractive drug candidates to treat bacterial infections [101]. Artificial inoculation of *S. aureus* in human nares was shown to be completely cleared by intranasal administration of MV-L endolysin of phiMR11, a phage specific for *S. aureus* [102]. Similarly, nasal/oral administration of CHAP domain of endolysin LysK eliminated *S. aureus* from nares of the infected mice [103]. The modular structure of the endolysins targeting Gram-positive bacteria is appropriate to evolve into efficacious drugs. A chimeric lysozyme ClyS, developed by fusing Ply Twort endolysin EAD and phi13 phage NM3CBD, reduced MRSA from nasal passage and showed better effect than mupirocin treatment [104, 105]. Furthermore, combination therapy of endolysin and antibiotics can be more effective in relieving MRSA infection [104]. Domain swapping strategy has also been used to replace the CBD of a phage endolysin PlyPSA and Ply187 to enhance the lytic ability when compared to their parental proteins [106]. Even the lytic spectrum of these enzymes can be broadened by engineering CBDs from different endolysins [106]. A *Staphylococcus* phage endolysin P128 is currently being investigated for intranasal administration against *S. aureus* in phase III clinical trials [107] and PlySs2 endolysin (CF-301) is also being tested against *S. aureus* for safety in phase I [108]. Recently, SAL-200 endolysin, derived from staphylococcal phage SAP-1, is the first intravenously administered lysin, which showed good tolerance with no serious adverse effects in phase I safety studies [109, 110].

In 2012, Lukacik et al. showed that the fusion of FyuA-binding domain of pesticin and T4 lysozyme utilizes FyuA for transport across the OM of Gram-negative *Yersinia pestis* [111]. This hybrid toxin killed *Yersinia* and *Escherichia coli* strains and also bypassed the pesticin immunity (PIM) [111]. Furthermore, Ply187-derived CD, when fused to non-SH3b CBD from phage phi13 endolysin NM3, protects mice against MRSA [112]. Endolysins harboring SH3b CBD were reported to ensure 100% survival when compared to oxacillin and vancomycin in bacteremia model [113]. An important vision harming disease called endophthalmitis, in which *S. aureus* colonizes the eye, was treated effectively in mouse model by chimeric endolysin Ply187AN-KSH3b [114]. Native CD and CWBD were joined via a linker to develop chimeolysins (e.g. Lys168-87, Lys170-87, B30-182-lyso, Ply187N-V12C and ClyR), which showed broad antibacterial spectrum [112, 115]. One such chimeolysin, ClyR, effectively lyses *Streptococcal* spp. (*S. pyogenes*, *S. agalactiae*, *S. dysgalactiae*, *S. equi*, *S. mutans*, *S. pneumoniae*, *S. suis* and *S. uberis*), *E. faecalis* and *S. aureus*, including MRSA [115]. The chimeolysin ClyR is also effective in killing *Streptococcus* mutans, which colonizes as biofilm on tooth surface [115].

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A shortcoming of natural endolysins is their inability to cross the OM of Gram-negative sp. (e.g. *P. aeruginosa*, *Salmonella typhimurium*, *Salmonella enterica*, *A. baumannii, E. coli, S. aureus* and *Bacillus subtilis*) [96, 97, 116]. Gram-negative bacteria have OM which is composed of lipopolysaccharide (LPS) and is only permeable to molecules smaller than 600 Da [117]. But recently, a Gram-negative endolysin SPN1S has shown to carry muralytic and glycosidic hydrolase activities in its alpha-helical structure [118]. To effectively penetrate OM of Gram-negative bacteria, endolysins have been fused to LPS-destabilizing polycationic peptides (PCNPs) to generate "Artilysins" [119]. The polycationic peptides can be fused to either N- or C-terminal of the endolysins but artilysins with N-terminal peptide are generally more effective [119]. Different LPS-destabilizing peptides, providing varying degrees of effectiveness, have been tested in artilysin constructs with polycationic peptide (PCNP) being the most effective one [119, 120]. The polycationic peptide punctures the LPS layer and facilitates the endolysin penetration into the OM, which subsequently degrades the PG layer [119]. An artilysin "Art-175" is a fusion product of KZ144 endolysin of *P. aeruginosa* phage phiKZ and sheep myeloid 29 amino acid peptide (SMAP-29) which can kill *P. aeruginosa* by more than 5 log in 30 minutes [119, 120]. Similarly, N-terminal fusion of PCNP to OBPgp276 endolysin (LoGT-001) of *P. fluorescens* phage OBD or PVP-SE1gp146 endolysin (LoGT-008) of *S. enterica* phage PVP-SE1 reduces *P. aeruginosa* by 4–5 log in 30 minutes [119]. Fusion of PCNP via C-terminal extended linker to OBPgp276 endolysin (LoGT-02) was as effective as N-terminal PCNP fusion [119]. Artilysin (Art-240; PCNP-λSa2lys endolysin) activity of greater than 3 log has also been demonstrated against Gram-positive bacteria (*S. agalactiae*, *S. dysgalactiae*, *S. pyogenes*, *S. uberis*, *S. suis*, *S. porcinus*, *S. gordonii*, *S. sanguinis*, and *S. viridans*) [121].

In addition, the protein transduction domains (PTDs), which facilitate protein transport across the eukaryotic membrane, have been used to engineer endolysin [94, 122, 123]. Fusion of endolysin with PTD is highly effective in reducing *S. aureus* burden in epithelial cell lines when compared to non-PTD endolysins [94, 123]. Catalytic peptides can also enhance the properties of a lysin as shown by fusing the Cecropin A peptide (residues 1–18) to the OBPsp279 lysin, which targets *A. baumannii* and *P. aeruginosa* during the phage growth [124]. More clinical trial data are needed to assess the safety and efficacy of these lysins.

9. Concluding remarks

Given the wide spread MDR bacteria and scarcity of new antibiotics in drug development pipeline, alternative options have to be explored urgently. As an alternative option, phage therapy is reattracting worldwide attentions. It is clear that phage therapy has several advantages in targeting against bacterial infections over conventional antibiotics [39, 125]: (1) phage is natural killer of bacteria that dictates its unique target specificity; (2) phage multiplies within bacteria host until host is lysed in a self-dosing manner; (3) phage shows efficacy to bacteria of MDR and (4) phage is environmentally friendly. Moreover, co-administration of phage or phage cocktail with antibiotics demonstrates synergistic effect over each individual treatment and increases antibiotics sensitivity from previous reports as reviewed in this chapter. However, as live virus, safety concern of phage therapy, due to the gap of deep understanding of phage-bacteria-human interaction network, is not easily cleared in the Western countries though former Soviet Unions accumulated a lot of empirically successful clinical reports in the nearly past 50 years.

Quality control of phage therapy based on Western medicine criteria has to be met. A small scale and strict quality control of a phage cocktail for treatment of *P. aeruginosa* and *S. aureus* infections was conducted in Belgium that included sequencing of whole phage genomes to verify the lack of toxin-encoding genes, confirmation of lytic phage property, lack of temperate phage, stability, removal of pyrogen, sterility and cytotoxicity [126]. This small-scale pilot study may set a foundation of standard in the Western countries for large-scale controlled clinical trials for phage therapy. Phage engineering can be employed to keep bacterial killing property but bypass lysis induced endotoxin release and related side effect [127]. More recently, human humoral immune response against phage therapy showed that anti-phage antibodies (Abs), including IgM, IgG and IgA, were detected in patient sera when staphylococcal MS-1 phage cocktail was used for treatment [128]. Interestingly, these anti-phage Abs did not compromise the final efficacy [128].

However, it seems that big pharmaceuticals are currently not interested in phage therapy, investment cost burden and patent filing may be another key considerations besides safety concern. To remove the worries from live virus-based therapy, phage-derived proteins (VAPGHs and endolysins) may become an option as these proteins also show the specificity and lytic efficiency against Gram-positive bacteria, albeit less efficient against Gram-negative bacteria due to the presence of OM cell wall.

Author contributions

DW conceived the topic of the study. All authors wrote the manuscript. LJ and AS contributed equally in writing the manuscript. DW revised the manuscript.

Funding

This work was supported by National Natural Science Foundation of China (Grant No.: 81572698) to DW.

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