


# Beyond antibacterials – exploring bacteriophages as antivirulence agents

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Shen, Yang ; Loessner, Martin J.

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# Beyond antibacterials – exploring bacteriophages as antivirulence agents

Yang Shen and Martin J Loessner

Life-threatening infections caused by multidrug-resistant bacteria are becoming increasingly difficult to treat. There is growing interest in exploiting bacteriophages (or phages) to combat bacterial infections. Phages often target bacterial surface structures that may also be important for virulence. Upon phage challenge, these molecules may be lost or modified, resulting in phage resistance and possibly phenotypical conversion. Importantly, possible trade-offs may include lower fitness, increased sensitivity to antibiotics and immune defense mechanisms, and virulence attenuation. Although evolution of phage-resistance may be difficult to prevent, the trade-off phenomenon carries potential for antibacterial therapy. Here we present some insights into the molecular principles and significance of this coincidental interplay between phages, bacteria, and immune cells, and discuss the prospect of developing phage-derived products as antivirulence agents.

## Address

Institute of Food, Nutrition and Health, ETH Zurich, Schmelzbergstrasse 7, 8092 Zurich, Switzerland

Corresponding author: Shen, Yang ([yang.shen@hest.ethz.ch](mailto:yang.shen@hest.ethz.ch))

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## Introduction

Phages are viruses that infect specific bacterial hosts. As natural predators of bacteria, they are the most abundant self-replicating organic entity in the biosphere, with an estimated  $10^{30}$  phage particles existing at any given moment [1]. This predator-prey relationship contributes to the evolution of bacterial populations by driving microbial diversity [2]. Phages are divided into two general categories: Lytic (virulent) phages begin replication immediately after cell infection, leading shortly to the degradation of host cells through lysis from within, mediated by dedicated peptidoglycan hydrolases (endolysins) [3]; while temperate phages can integrate their genetic

information into the bacterial chromosome, remaining in place until the conditions for producing progeny are met [4].

The first step of infection by a tailed phage (*Caudovirales*) is adsorbing to a susceptible host cell, mediated by receptor-binding proteins (RBPs), which are located on the distal end of the tail apparatus [5]. Therefore, these proteins are also known as tail spikes or fibers, which are the major determinant of host range by targeting specific surface-accessible receptors distributed in a genus-specific, species-specific or even strain-specific manner. The host cell wall-associated receptors range from macromolecules (proteins and polysaccharide) to organelles (flagellum and pili) [6]. Given their extraordinary specificity and bacteriolytic activity, phages represent highly attractive antibacterial agents for biotechnological [7,8] and therapeutic applications [9,10].

Bacteria can evolve resistance to phage by modifying their surface receptors through genetic mutation in response to phage-driven selective pressure. Phage-encoded lytic enzymes (e.g. endolysins and depolymerases) are capable of degrading these binding receptors. Notably, these receptors may also serve as virulence factors contributing to pathogenicity, and modification of these structures as a result of gaining phage resistance often come at a cost and may lead to attenuation of fitness and virulence, such as defective in growth, biofilm formation, and colonization, as well as more susceptible to antibiotic treatment and immune defense. With this in mind, phages and some of their lytic enzymes may be exploited as antivirulence agents to prevent or even treat bacterial infections, adding to their bactericidal properties. In this review, we explore this idea through an overview of bacterial host receptors for phage recognition, how bacteria modify their receptors to become recalcitrant to phage infection, and the possible phenotypical consequences. We discuss recent advances and implications of developing phage-encoded enzymes as antivirulence agents. Finally, we highlight the synergistic potential of using phage-derived agents in combination with antibiotics and host defense to combat bacterial infections that are difficult to treat by conventional therapy.

## Bacterial cell surface macromolecules as phage receptors

In Gram-positive bacteria, a diverse array of host cell receptors have been identified, including flagellum filament, membrane proteins, and surface-associated

glycopolymers (Figure 1). For a comprehensive summary, we refer the reader to a recent review on this topic [5]. Recent efforts have uncovered several types of glycopolymers-based receptors for Gram-positive infecting phages, such as lactococcal cell wall polysaccharide (CWPS) [11], streptococcal CWPS [12], enterococcal polysaccharide antigen (Epa) [13\*\*], *Clostridium* capsular polysaccharide [14], *Listeria* peptidoglycan (PG) [15] and PG-anchored wall teichoic acid (WTA) [16].

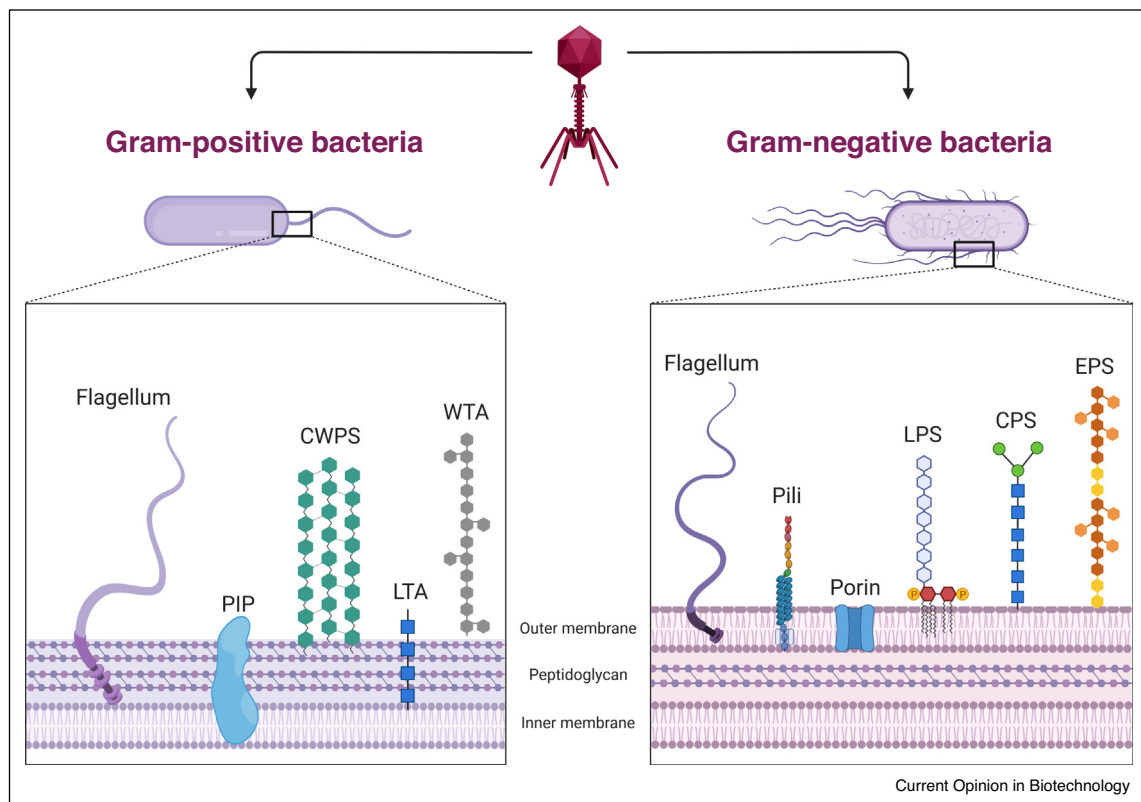
The primary phage receptors of Gram-negative bacteria are surface-associated glycopolymers, including LPS (lipopolysaccharide), CPS (capsular polysaccharide), and EPS (exopolysaccharide). For a detailed overview of Gram-negative phage receptors, we refer the reader to the review [17\*\*]. Phage-expression of CPS has been demonstrated to mediate the phage adsorption in *Bacteroides thetaiotaomicron* [18] and *Campylobacter jejuni* [19]. While a variety of *Salmonella* and *Escherichia* phages are known to be flagellotropic and bind to flagellin proteins upon initial adsorption, a recent study showed that the bound phages can move along the flagellum by helical

movement until they reach cell wall-associated receptors (e.g. LPS) where irreversible binding occurs [20]. Other filamentous appendages, namely pili, may also represent important structures for phages to attach and infect Gram-negative pathogens, such as *Vibrio cholerae* [21] and *Stenotrophomonas maltophilia* [22]. In addition, a variety of outer membrane proteins (e.g. porins OmpC, OmpF, and TolC) have been identified as the secondary receptor for *Escherichia*, *Samonella*, and *Vibrio* phages [17\*\*,23].

### Receptor-mediated phage resistance often comes at a cost for fitness and virulence

Bacteria are under enormous evolutionary pressure based on natural phage-mediated predation. Therefore, they have developed numerous sophisticated defense mechanisms, such as restriction-modification, CRISPR-Cas, and other abortive infection systems to cope with viral infection [24]. One of the most common types of phage resistance in bacteria involves modification of phage receptors by mutation of genes responsible for the biosynthesis and/or assembly of these receptors, thereby preventing phage adsorption. Notably, these receptors

Figure 1



Schematic illustrations of Gram-positive (left) and Gram-negative (right) cell surface-associated virulence factors that act as phage receptors. On the left, a rod-shaped and flagellated Gram-positive bacterium is shown with surface-exposed PIP (phage infection proteins), CWPS (cell wall polysaccharide), LTA (lipoteichoic acid), and WTA (wall-teichoic acid). On the right, a rod-shaped, flagellated and piliated Gram-negative bacterium is depicted with surface-associated porin, LPS (lipopolysaccharide), CPS (capsular polysaccharide), and EPS (exopolysaccharide). Selective pressure mediated by phages can modify these receptors through genetic mutation, which results in the trade-off between fitness cost and virulence attenuation. Various colors of circles, hexagons, and squares represent different sugar moieties.

also function as virulence factors involved in various pathogenicity pathways, such as colonization in the host, evasion of the host's immune response, biofilm formation, toxic shock, and host cell invasion [25]. Hence, phage resistance may result in trade-offs to bacterial fitness and virulence, and may be exploited to improve treatment outcomes [26].

### Fixed surface alteration by genetic mutations

Several phage resistance mechanisms have been described to create such trade-offs. For example, loss or modification of LPS structures has been shown to diminish phage infection and reduce virulence [27]. Targeting pili as virulence factors via phage therapy has been proposed as a potential antivirulence approach that could modify bacterial population while selecting pili-deficient strains, which often cause less severe pneumonias during acute pulmonary infection [28]. Predation by *Pseudomonas aeruginosa* phages that use type IV pilins as receptors selects for strains with glycosylated pili, to block phage infection [29\*\*]. In a separate study, *P. aeruginosa* phage resistance was found to be associated with the reduction in efflux pump efficiency [30], a trade-off affecting antibiotic resistance. This type of directed evolution has the potential to prevent the emergence of antibiotic resistance or even possibly resensitize bacteria to antibiotics [31].

In Gram-positive bacteria, a recent study showed that enterococcal phages require Epa for adsorption, as phage predation favors mutation in nonconserved *epa* genes that are located in the gene cluster encoding enzymes for Epa production [32]. Moreover, these Epa mutants were found to be deficient in intestinal colonization, and fail to expand its population upon antibiotic treatment due to its increased susceptibility to cell wall-targeting antibiotics [13\*\*].

We have recently demonstrated a striking trade-off between phage resistance and virulence attenuation in the pathogenic serovar 4b *Listeria monocytogenes* strains [33]. Here, challenge by phages selects for surviving clones that specifically feature a loss of galactose (Gal) from the WTA polymers, by mutations in genes involved in WTA galactosylation. Interestingly, similar mutations were also found to occur in the serovar 4d or 4e strains isolated from the environment. The loss of Gal not only prevents phage adsorption, but also features a complete loss of the surface-associated invasion protein Internalin B, the inability to form actin tails required for cell-to-cell spread, resulting in a massive virulence attenuation *in vivo*. These phage-insensitive bacteria are unable to interact with mammalian cMet and gC1q-R host cell receptors, which normally trigger bacterial uptake upon interaction with InlB. In a follow-up study, we identified the genes responsible for galactosylation of teichoic acids in the important serovar 4b strains, and demonstrated that

galactosylated WTA is solely accountable for phage adsorption, InlB surface presentation, and cellular invasiveness [34\*\*]. Collectively, these findings suggest a trade-off between phage resistance and *Listeria* virulence.

### Phase-variable modification of phage receptors

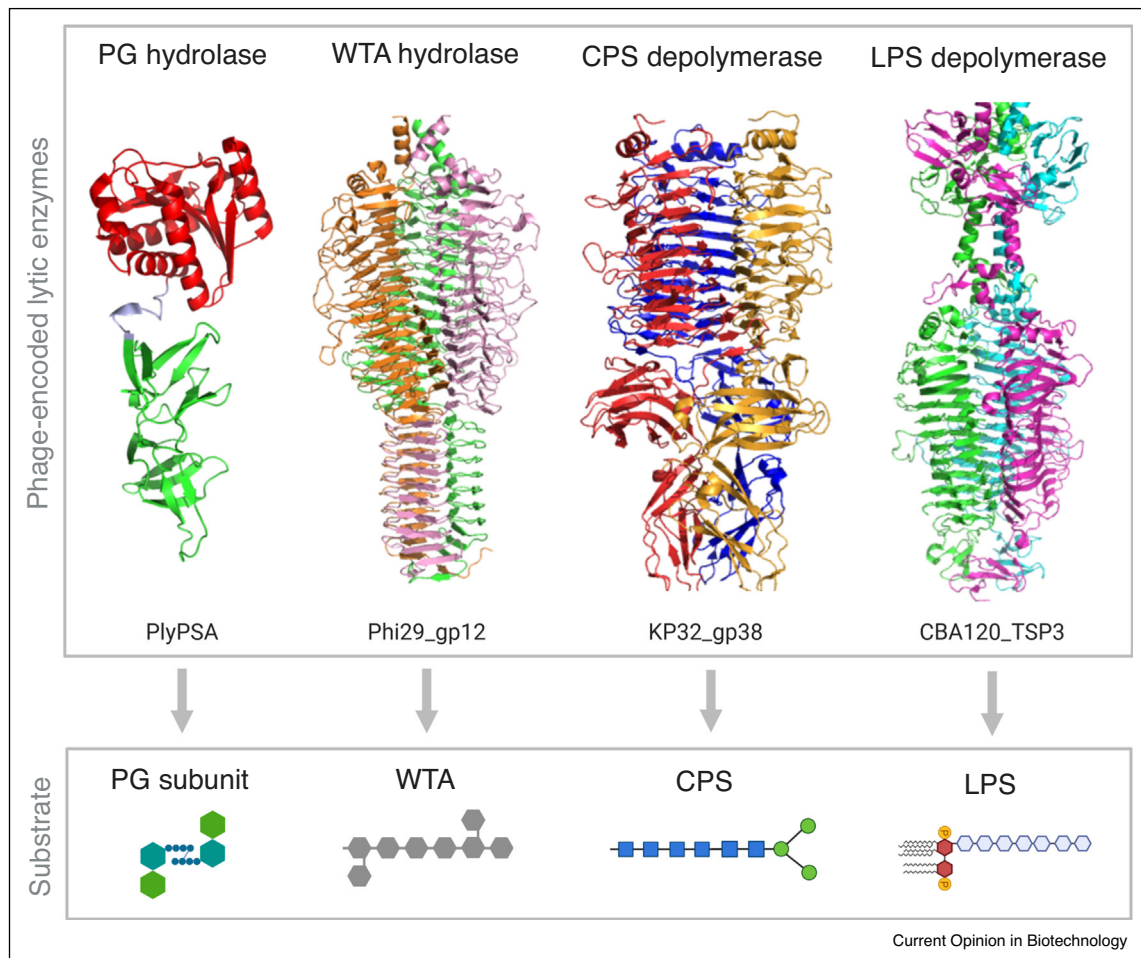
Besides genetic mutations induced by phage predation, non-genetic modification of phage receptors in a phase-variable manner has been reported to confer phage resistance and alter host-pathogen interaction. Phase-variable expression of CPS has been shown to modulate phage sensitivity in *B. thetaiotaomicron* [18] and *C. jejuni* [19]. Transient truncation of LPS in length is known to be associated with phage resistance in *P. aeruginosa* [35]. The Gram-positive pathogen *Staphylococcus aureus* exhibits decreased levels of WTAs in the cell wall, which appears to negatively affect the bacterial ability to form skin abscesses, demonstrating a novel phase-variable virulence system [36]. Altogether, such transient control systems demonstrate another very interesting type of trade-off between phage resistance and host colonization.

### Phage-derived lytic enzymes as antivirulence agents

Phages possess a set of lytic enzymes that are capable of degrading bacterial surface glycopolymers that function as phage receptors and virulence factors. These enzymes are generally referred to as endolysins (peptidoglycan hydrolases) and depolymerases (including WTA hydrolases, CPS, or EPS endoglycosidases or exoglycosidases). The architectures of representative enzymes and their substrates are depicted in Figure 2.

**Endolysins** are peptidoglycan degrading enzymes encoded by almost all phages and are produced within the infected host bacterium at the end of the multiplication cycle [3]. The exposed cell wall of Gram-positive bacteria allows endolysins to exert bacteriolytic activity in a highly specific and efficient manner when adding externally without damaging the non-target bacteria. To digest the PG, endolysins from a Gram-positive background have evolved to utilize a modular design in which catalytic activity and cell wall recognition are separated into two types of functional domains termed enzymatically active domain (EAD) and cell wall binding domain (CBD). EADs feature 4 major categories based on their cut sites within the PG: muramidases and glucosaminidases cleave bonds within the disaccharide backbone; *N*-acetylmuramoyl-L-alanine amidases cut the amide bond between MurNAc and peptide moieties; and endopeptidases cut within the peptide stems. CBDs confer specificity (at a genus, species, or even strain level) to endolysins by targeting the carbohydrate epitope in the cell wall, which can be parts of the PG itself [37] or cell wall-associated glycopolymer [38]. Although endolysins are predominately applied for direct lysis of target bacteria,

Figure 2



Representative phage-encoded lytic enzymes that are able to degrade bacterial surface-associated virulence factors.

The cartoon representations from left to right: *Listeria* phage endolysin PlyPSA (PDB ID, 1XOV) digests the peptidoglycan (PG); *Bacillus* phage appendage protein Phi29\_gp12 (PDB ID, 3GQA) acts as a wall-teichoic acid (WTA) hydrolase; *Klebsiella* tailspike protein KP32\_gp38 (PDB ID, 6TKU) functions as a capsular polysaccharide (CPS) depolymerase; *E. coli* tailspike protein CBA120\_TSP3 (PDB ID, 5W6F) is a lipopolysaccharide (LPS) depolymerase. The respective substrate for each enzyme is shown below the cartoon structure. Various colors of circles, hexagons, and squares represent different sugar moieties.

one should not overlook their potential to degrade the PG involved in the pathogenesis and immune evasion of different human pathogens, specifically *O*-acetylation of PG as a novel target for antivirulence therapies [39].

**Depolymerases** are tail spike proteins (TSPs) that degrade the highly immunogenic CPS or biofilm EPS to access the primary receptors for phage infection [40]. They normally form a stable homotrimer functioning as both receptor-binding proteins for polysaccharide recognition and glycanases. Phages that infect *Escherichia* spp. encode glycosidases (TSP1 and TSP3) [41] to degrade LPS. In *Pseudomonas* spp. phage LKA1, the encoded depolymerase Gp49 contains an O5 serotype-specific polysaccharide lyase, which has been shown to disrupt biofilm and reduce virulence, while sensitizing bacteria to serum complement

activity [42]. *Klebsiella* phage-borne depolymerases Dep42, KP32Gp37, and KP32Gp38, have been demonstrated to degrade specific serotypes of bacterial CPS and inhibit biofilm formation [43]. Degradation of *Klebsiella* capsules renders enhanced complement-mediated serum killing and phagocytic clearance, thereby increasing the lifespan of infected animals [44]. Similar EPS depolymerases have been reported in phages infecting *Erwinia amylovora* [45] and *Providencia stuartii* [46]. These data support the development of these capsule-targeting depolymerases as promising antivirulence agents (Table 1).

To date, only scarce information is known about putative depolymerases encoded by Gram-positive phages. The pre-neck appendage protein of *Bacillus* spp. phage phi29\_gp12 mediates the irreversible cell wall attachment



Table 1

## Selected reports on phage-derived enzymes as antivirulence agents

Bacterial species	Phage type	Phage enzyme	Substrate specificity	Antivirulence property	Ref.
<i>Pseudomonas aeruginosa</i>	LKA1	Gp49	B-band LPS	Disrupt biofilm, reduce virulence, and sensitize bacteria to serum complement activity	[42*]
<i>Klebsiella pneumoniae</i>	SH-KP152226	Dep42	CPS	Inhibit biofilm formation and degrade formed biofilms.	[43]
<i>Klebsiella pneumoniae</i>	KP32	Gp37 and Gp38	Serotype K3 and K21 CPS	Enhance complement-mediated serum killing and phagocytic clearance	[44]
<i>Providencia stuartii</i>	Stuart	Gp52	EPS	Render drug-resistant bacteria susceptible to serum killing	[46]
<i>Bacillus subtilis</i>	Φ29	Gp12	Glycerol-type WTA	Depolymerize glycosylated WTA chain	[47]
<i>Staphylococcus aureus</i>	vB_SepiS-philPLA7	Dpo7	EPS	Disperse staphylococcal biofilms	[48]

and can degrade cell wall teichoic acids with phosphodiesterase activity [47]. Its homolog Dpo7 (identified in a staphylococcal phage) was shown to exhibit biofilm-degrading activity by acting on the surface EPS structures [48]. Since both WTA and EPS represent important virulence factors for many pathogenic bacteria, degradation of these matrix-like structures may facilitate the penetration of antibiotics and the clearance by immune systems, resulting in increased therapeutic efficacy.

In summary, depolymerases and endolysins feature modular structures and combine different conserved and variable modules conferring their enzymatic activities and host specificities. From a therapeutic point of view, these enzymes hold promises to be developed as tailor-made antibacterial and antivirulence agents. For a detailed examination of published endolysins and depolymerases, see previously published reviews [3,40].

### Phage-derived antivirulence strategy: possible synergy with other antibacterials

With the increasing number of studies or clinical trials using phages or phage lytic enzymes as bacteriolytic therapeutics, there is growing interest in developing them as virulence-specific antibacterials for infection control. Unlike conventional drugs that directly kill bacterial cells or inhibit their growth, the antivirulence strategy aims to disarm the pathogens, rendering them less virulent and fit, or more susceptible to antimicrobials or innate host defense. A similar strategy has recently been described as ‘phage steering’, which could be advantageous when treating infections [31].

### Phage-antibiotic or phage enzyme-antibiotic synergism

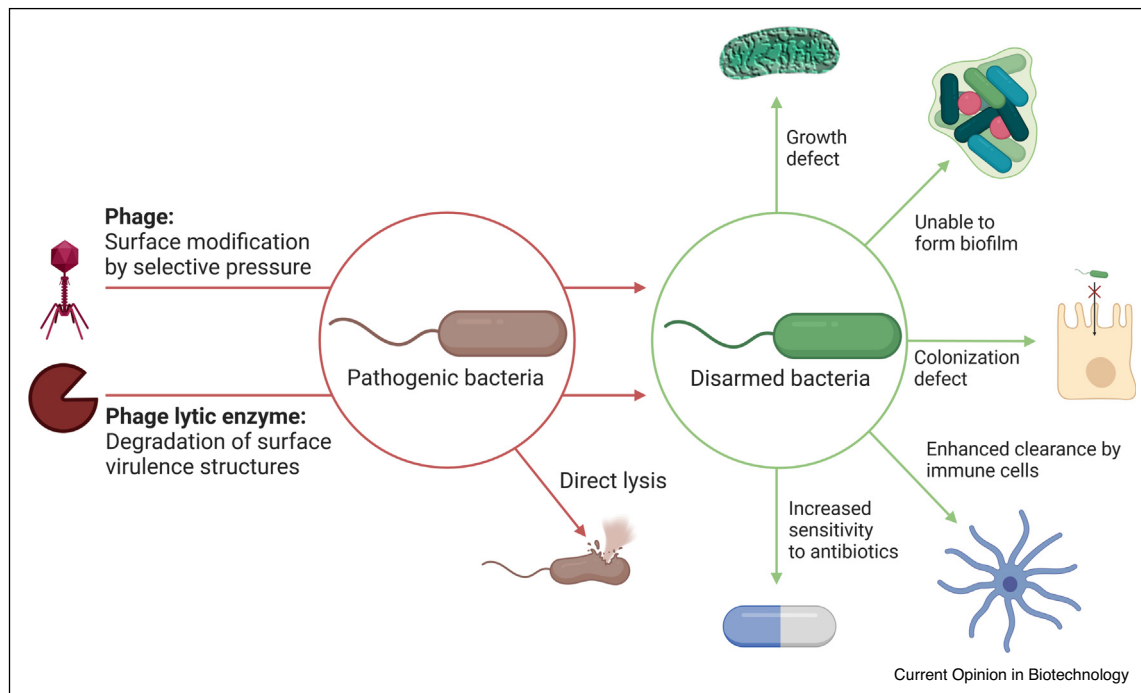
A combination of phage and antibiotics has been considered a preferred choice in phage therapy [49]. Yet, the mechanism drives the synergy remains unclear. Previous work has attributed the synergy to the selective pressures and resistance costs that resensitize bacteria to antibiotic treatment [13\*\*,30,32,33], as antibiotic susceptibility and phage resistance may be based on the same phage

receptor, such as the glycopolymers or the proteins of efflux pump. Recently, a patient with life-threatening *Acinetobacter baumannii* infection has been successfully treated using a personalized phage cocktail in combination with minocycline [10]. As for phage-encoded enzymes, synergy with standard-of-care antibiotics has also been demonstrated for *Klebsiella* depolymerase Dep42 [43] and staphylococcal endolysin CF301 [50]. The latter is primarily attributed to the bacteriolytic action of endolysins, whereas the former is due to the degradation of the CPS layer, permitting the increased penetration of the membrane-acting compound polymyxin. These studies substantiate that phage-mediated modification on the bacterial surface can be clinically beneficial and enable the recycling of ineffective antibiotics back into effective drugs.

### Phage-host or phage enzyme-host defense interaction

Phages can modulate both innate and adaptive immunity via interaction with mammalian immune systems, thereby exhibiting profound effects on the outcome of bacterial infections [51]. The concept of ‘immunophage synergy’ has recently arisen in a study investigating the interplay between phage, *P. aeruginosa*, and immune cells using both *in vivo* and computational models [52]. The data indicate that successful phage therapy is supported by innate immune components, specifically neutrophils to kill phage-sensitive and emerging phage-resistant pathogens. Phage-resistant *K. pneumoniae* mutants with deficient CPS synthesis were found to be more sensitive to macrophage-mediated phagocytosis [53]. Some contrary data show phages may also mislead the immune system, thereby preventing the clearance of *P. aeruginosa* infection in an open-wound setting [54]. In this study, a filamentous temperate phage was found to be integrated into the bacterial genome and to suppress the innate immune response. Similar findings were revealed in a separate study that phages can stimulate a specific immune response, which worsened inflammatory bowel disease pathogenesis [55]. Because of this previously unrecognized complexity of host–microbe–phage

Figure 3



Approach to develop phages or phage-encoded glycan degrading enzymes as antivirulence agents for combating antibiotic-resistant infections. The graph illustrates the fitness trade-offs promoted by surface virulence factor mutation upon phage predation, and the degradation of virulence glycans by the application of phage lytic enzymes. The modes of action are: (1) direct lysis of target bacteria; (2) disarming pathogenic bacteria with surface modification by selective pressure or enzymatic treatment. The attenuated bacteria frequently bear a fitness defect, which may render them unable to form biofilm, defective in colonization, or more susceptible to antibiotics and immune cells.

interactions, further mechanistic details are needed to develop specific phage-immune combinatorial therapies for the treatment of bacterial infections. In addition, several phage-encoded depolymerases act synergistically with serum-mediated killing, complement activity, and phagocytosis [42<sup>\*</sup>,44,46], as these enzymes primarily target surface glycopolymers which modulate the interaction between bacteria and immune system. These exciting synergies between depolymerases and the immune defense pave promising avenues to investigate how depolymerases enhance antibacterial therapies as antivirulence agents.

### Conclusions and perspectives

Until now, phage therapy was thought to work simply by reducing bacterial numbers, in a way to known antibiotic therapies. However, treatment failure occurs when bacteria are able to develop phage resistance during phage administration [56]. Several strategies have thus been proposed that go beyond simple phage monotherapy in order to preclude resistance, such as multi-phage cocktails, phage engineering, and approaches combining phages with antibiotics or the immune defense. That being said, phage resistance may also be anticipated and used as part of a therapeutic strategy (Figure 3). This

involves harnessing the evolutionary trade-off that occurs when phage-resistance is accompanied by increased sensitivity to conventional antimicrobials or the immune system or attenuated virulence. The same is true for the aforementioned phage lytic enzymes which are capable of disarming pathogens in addition to their direct killing activity.

Although phages or their lytic enzymes represent promising antivirulence biologics, several challenges exist towards possible clinical application: (1) avoid unwanted prophage-encoded virulence factors and interactions with the immune system; (2) fine-tune the selection pressure and coevolution between the specific phages and their bacterial hosts; (3) better mechanistic understanding of the synergy between phage (or phage enzymes) and antibiotic (or immune defense) to achieve the ideal outcome of combination therapies; (4) better understanding of phage-resistance mutations that pleiotropically complicates the trade-off [57<sup>\*\*</sup>]; (5) improve stability and pharmacokinetics of recombinant phage enzymes. We have no doubt that following these new directions in phage therapy research will greatly aid the development of effective phage-based antivirulence agents.

## Conflict of interest statement

Nothing declared.

## CRedit authorship contribution statement

**Yang Shen:** Writing - original draft, Writing - review & editing, Visualization. **Martin J Loessner:** Writing - review & editing.

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