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Bacteriophages and Phage-Derived Proteins – Application Approaches

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Abstract: Currently, the bacterial resistance, especially to most commonly used antibiotics has proved to be a severe therapeutic problem. Nosocomial and community-acquired infections are usually caused by multidrug resistant strains. Therefore, we are forced to develop an alternative or supportive treatment for successful cure of life-threatening infections. The idea of using natural bacterial pathogens such as bacteriophages is already well known. Many papers have been published proving the high antibacterial efficacy of lytic phages tested in animal models as well as in the clinic. Researchers have also investigated the application of non-lytic phages and temperate phages, with promising results. Moreover, the development of molecular biology and novel generation methods of sequencing has opened up new possibilities in the design of engineered phages and recombinant phage-derived proteins. Encouraging performances were noted especially for phage enzymes involved in the first step of viral infection responsible for bacterial envelope degradation, named depolymerases. There are at least five major groups of such enzymes – peptidoglycan hydrolases, endosialidases, endorhamnosidases, alginate lyases and hyaluronate lyases – that have application potential. There is also much interest in proteins encoded by lysis cassette genes (holins, endolysins, spanins) responsible for progeny release during the phage lytic cycle. In this review, we discuss several issues of phage and phage-derived protein application approaches in therapy, diagnostics and biotechnology in general.

Keywords: Bacteriophage application, endolysins, holins, phage-encoded proteins, polysaccharide depolymerases, spanins.

1. INTRODUCTION

At the beginning of the 20th century, Frederick Twort [1] and Felix d'Hérelle [2] as bacteriophage discoverers, proposed phage utilization as natural antibacterial agents. At that time, at least seven commercial phage preparations to combat skin abscesses, ulcers and other topical infections, had been manufactured. Unfortunately, the lack of knowledge of phage biology, non-standardized manufacturing procedures and storage of phage products made them considerably unstable, which gave rise to reasonable distrust among physicians and clinicians [3, 4]. Studies on bacteriophages in Western Europe and the United States were discontinued, when era of antibiotics started and when penicillin discovery by Alexander Fleming became a fact. Drug therapy introduced in the large-scale was remarkably successful, therefore, the interest in phage research/applications has been diminished. However, drug extensive application have resulted in the massive increase of bacterial drug resistance and the scientists have turned back to the idea of phage utilization as potential alternative tool against pathogens. Numerous publications mostly from Eastern Europe and the Soviet Union have proved the efficacy of phages in eradication of most common bacteria causing various infections in humans and animals [5-10]. Nowadays, endolysins, exopolysaccharidases, and other phage-encoded proteins, are extensively explored as effective natural antibacterials in food product preservation, in animal feeding, and in plant cultivation [11-14]. Due to the high specificity of host receptors' recognition by phage particles, bacterial viruses have also been successfully utilized as a clinical diagnostic tool and in detection of potential foodborne pathogens [15, 16]. The rapidly developing synthetic biology creates new possibilities in the design of genetically modified phages or recombinant phage-derived particles to improve their application in therapy, diagnostics and biotechnology [17, 18]. In this review, we concentrate on current data regarding emerging approaches in use of unmodified and engineered bacteriophages and phage-derived products.

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2. BACTERIOPHAGES

Bacterial viruses (phages) can be found in each environment inhabited by their bacterial hosts. In aquatic systems, the abundance of phage population was estimated as 10^4 to 10^8 virions per ml, and as 10^9 virions per 1 g in the soil and sediment particles [19]. Currently, over 5500 different bacteriophages, having genetic information encoded by dsDNA, ssDNA, ds RNA and ssRNA, have been discovered, [20]. Phage life cycle can be carried out by several schemes: lytic, lysogenic, pseudolysogenic and chronic [19]. The intensive research on phage biology and genetics allows for increase the phage potential in different areas of human activity. The main promises are associated with phage and phage-encoded proteins utilization in: (i) phage typing; (ii) phage therapy; (iii) food decontamination; (iv) medical devices disinfection;

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(v) bacterial detection; (vi) drug delivery (vehicles) and (vii) molecular biology. The novel and old approaches in bacterial diagnostics (typing, detection, separation) have been recently presented in several papers [15, 16, 18, 21, 22]. Generally, diagnostic techniques have been divided into three types: (i) phage amplification assay – detection of living cells only; (ii) phage as a recognition agent; and (iii) detection of intracellular components after phage-induced lysis. Described methods include the utilization of: (i) natural phages in standard plating assay and determination of plaque forming units, or phage detection by labelled anti-phage antibodies; (ii) genetically modified phages carrying additional reporter genes (LRP, GFP, β-Gal) or tagging peptides for further staining, and detection based on bioluminescence, fluorescence, enzymatic reactions or by electrochemical and mass sensors; (iii) measurement of ATP or bacterial enzymes (α- and β-Gal) release, and analysis of culture media composition changes. The particular characteristics and possibilities in phage-derived protein application in bacterial diagnostics, as well as in bacterial eradication, will be discussed below following the section dedicated to virulent particles.

The general characteristics of phage utilization in bacterial infection treatment are summarized in (Table **1**) and discussed in detail in the following description.

The most abundant group of lytic phages belong to tailed dsDNA viruses (the *Caudovirales* order), thus the main interest in phage application, past and present, has focused on three families: (i) *Myoviridae*; (ii) *Siphoviridae*, and (iii) *Podoviridae* [3, 23, 24]. The high efficiency of lytic phage therapy is based on its two principal properties: specificity in recognition of appropriate complementary receptors on susceptible host, and bacterial cell disintegration after a short viral life cycle leading to fast eradication of the targeted pathogen [23, 25-30].

Most bacteriophages may infect limited number of strains among a single bacterial species. The ability to affect is conditioned by highly specific interaction between host cell surface receptors and phage attachment structures [19, 31]. Phages may recognize several bacterial cell structure components as their receptors: outer membrane proteins, peptidoglycan (PG), teichoic acids, oligosaccharides, lipopolysaccharide (LPS), capsule, flagellum, type IV fimbriae, and sex pilus [28, 29]. The limited host range of individual phage may be considered as an advantage because phages eliminate only the targeted strain, protecting normal flora colonizers unrelated to pathogen species.

Nowadays the accessibility and usability of synthetic biology allow us to modify natural phages or design engineered viruses exhibiting desired attributes. Lu and coworkers [32] have presented lytic *Enterobacteria* phage T7 expressing biofilm-degrading enzyme enable to hydrolyse exopolysaccharide compounds (EPS) in bacterial biofilm during propagation. Such modified phage could be applied in eradication of monoculture or as phage-cocktail to treat mix-cultured biofilm. The primary concern for using engineered organisms is that it may affect the balance between natural viruses and bacterial hosts in the environment. However, Gladstone and co-authors [33] have proved that from an evolutionary point of view engineered phages carrying additional genes (for example of EPS depolymerase) lost the ability to compete with non-modified phages specific to the same host, where the latter could propagate freely and much faster on host cells in which EPS had been degraded by phage enzyme originated from the engineered competitor.

The virulence capacity of lytic phages conditions the antibacterial efficacy. Phage generation time, including the duration of efficient adhesion, latent period and progeny release, is an important element. The second aspect is the rate of phage population growth, what means the number of phage particles formed during one life cycle. The high adsorption rate to particular bacteria, large burst size, and short generation time define a strong antibacterial efficacy. Nevertheless variation of certain conditions influence on phage virulence. One of them is the current state of the bacterial culture, because predators may propagate most intensively on exponentially grown bacterial population. The second aspect is the multiplicity of infection (MOI), which is fundamental for phage titre-dependent killing [34-36]. Phage titre increases only in the presence of bacterial host, therefore the efficient concentration of phage particles may be achieved and maintained at the site of infection, until it is needed. Because of these features, bacteriophages are named as self-replicating antibacterial agents [37]. This advantage allows the reduction of curative doses. Unfortunately, lytic phage propagation sometimes leads to undesirable consequences. Rapid release of cellular toxins or disintegration of Gram-negative outer membrane in a short period may result in a systemic inflammatory response and serious side effects on the host [38].

These concerns have led researchers to explore non-lytic filamentous phages replicating in bacteria by permanent slow release of progeny, without cell lysis [39, 40]. Additionally, ssDNA phages are accessible objects for gene manipulation. There are several studies discussing the application of natural and modified *Enterobacteria* phage M13, Ike and fd and *Pseudomonas* phage Pf3 [17, 40-43]. The group of Hagens [40, 42] have proposed the utilization of modified M13 and Pf3 filamentous phages to overcome problems related to sudden bacterial lysis and to enhance antibacterial efficacy of phages. The researchers have designed phages that carry additional genes of the restriction endonuclease or holin system, causing bacterial genome degradation or inner membrane dysfunction, respectively, but preventing cell disintegration. Engineered phage Pf3 was designed in two versions: replicating non-lytic and non-replicating. The latter version prevented undesirable release of genetically modified viruses to the environment, while forcing the application of high MOI. Lu and Collins [44] have proposed to combine standard drug treatment with engineered antibiotic-enhancing phage M13mp18 carrying the *lexA3* gene, a repressor of the SOS bacterial response. It turned out that such a combination significantly enhanced the efficacy of ciprofloxacin, gentamicin and ampicillin in drug-resistant strains, regardless of antibiotic mode of action. Similar improvement have been obtained by the application of M13mp18 carrying other genes encoding crucial elements of bacterial cell biology (SoxRS – responsible for cellular susceptibility to superoxide; CrsA – regulator of glycogen synthesis; OmpF – an outer membrane protein conditioning drug penetration). M13 phages designed by Lu and Collins [44] were able to improve antibiotic activity against drug-resistant strains,

Table 1. Major Features of Phages Applied As Antimicrobial Agents.

Phage Type	Characteristics	Advantages	Limitations
Natural phages			
lytic	dsDNA *Caudovirales* bacteriolytic; phage titer-dependent killing; lytic efficacy: MOI, burst size, growth rate; effective on growing cells	self-replicating agent; concentration increase at the infection site; low MOI is usually sufficient	endotoxin (LPS) and other toxins release during cell lysis possible; inflammatory response may occur
non-lytic	ss DNA *Inoviridae* *Enterobacteria* phage M13 *Pseudomonas* phage Pf3 filamentous phages; do not lyse the host during progeny release	self-replicating agent; prevention of endotoxin (LPS) and other toxins release during cell lysis	no visible changes in viability of infected bacterial culture; relatively fast occurring of resistance by modification/loss of pili receptors
temperate	dsDNA *Siphoviridae* *Pseudomonas* phages DMS3, MP22, D3112 integration into bacterial genome; do not lyse the host during lysogenic infection	inhibition of bacterial virulence factors (bacterial group motilities, biofilm formation); host gene disruption by phage integration possible; prevention of endotoxin (LPS) and other toxins release during cell lysis	possible threats of unexpected consequences related to random integration into bacterial chromosome (horizontal gene transfer)
Genetically modified phages designed to reduce endotoxin release and to enhance antibacterial potency			
lytic	ds DNA *Podoviridae* *Enterobacteria* phage T7	self-replicating agent; carrying additional genes of biofilm-degrading enzyme	self- replicating agent with concern of recombinant phage release to environment;
non-lytic	ss DNA *Inoviridae* *Enterobacteria* phage Ike and fd *Enterobacteria* phage M13 *Pseudomonas* phage Pf3	self- or non-replicating agent; receptor domain modification extending host range; carrying additional genes of: toxic proteins, restriction endonucleases, holins, repressors of SOS system or other factors enhancing antibiotic activity	self- replicating agent with concern of recombinant phage release to environment; non- replicating agent needs high MOI value ($\geq$ 1000); relatively fast occurring of resistance by modification/loss of pili receptors;
temperate	ds DNA *Siphoviridae* *Enterobacteria* phage λ	phage display system, gene transfer, bio-detection, bio-control, phage vaccines, therapeutic binding agents; specificity similar as antibodies, used as inhibitors or agonists	non- replicating agent, immunogenic

persistent cells and biofilm-forming cells. Another study dealt with problems associated with the very limited spectrum of activity found in natural phages, related to high specificity of receptor recognition. Marzari has created the different set of proteins forming pili-recognizing phage fibres, made of two filamentous phages Ike and fd, resulting in an extended host range of the engineered phage. A very interesting approach was the idea of natural temperate phages application for modification of bacterial virulence, presented in some reports [45-47]. By integration into the bacterial genome, temperate *Pseudomonas* phages DMS3, MP22, D3112 were able to inhibit the expression of virulence factors such as bacterial group motility (swarming and twitching motility) or biofilm formation, which in consequence significantly reduce the mortality of *Pseudomonas*-infected animals. This phenomenon may be related to the host gene disruption by phage integration, CRISPR/Cas system interaction or to mechanisms independent of host background. Another way, to use temperate phages, has been described in detail in a recent review focusing on lambda phage display systems [48]. In that paper, the authors have discussed the advantages and capabilities of phage display utilization as a convenient tool for gene transfer, vaccine design and delivery, as well as bio-sensors, bio-detectors and bio-control agents.

The application of phages as antimicrobials, regardless of the nature of the virus (lytic, non-lytic, temperate, engineered), must always take into consideration the host anti-phage resistance mechanisms. In the phage-bacteria co-evolution, the latter has developed numerous adaptive strategies preventing viral infection. The anti-phage resistance mechanisms have been described recently in several comprehensive reviews [14, 49, 50]. There are four major types of mechanisms blocking phage propagation at crucial life cycle steps: (i) inhibition of phage adsorption; (ii) the restriction-modification (RM) system and clustered regularly interspaced short palindromic repeats (CRISPR) for phage DNA degradation; (iii) the superinfection exclusion system (Sie)

preventing DNA integration; and (iv) the abortive infection system (Abi) blocking phage transcription, translation or virion assembly. The most common resistance mechanism is based on phage adsorption prevention, which may be achieved by loss, modification or masking of a targeted bacterial receptor. Phages as a permanent partner of bacteria have been evolving simultaneously and adapt to new receptors or produce specific enzymes degrading saccharides that mask primary receptors (discussed below).

Anti-phage bacterial resistance can be acquired by mutation and selection, or/and horizontal gene transfer. The temperate phage acquisition or conjugation process may lead to acquisition of resistance genes. Finally, protecting mechanisms can be shed vertically from parental to daughter cells. Induction and propagation of temperate phage or the plasmid transmission condition the horizontal dissemination of resistance genes [19]. Several papers have reported the prevalence and probability of phage resistance development and it varied from a low-frequency level (10^{-8}) during phage therapy, to 10^{-4}–10^{-8} in some *in vitro* analyses [51-53]. The discrepancy between clinical observations and *in vitro* results can be attributed to the immune system activity, particular phage virulence or environmental condition variation [17, 54, 55]. To prevent possible resistance development among bacterial population, the phage cocktails usually composed of 3-5 types of viruses are applied in routine phage therapy [19, 38]. The cocktails are the most often designed to contain phages infecting through different cell receptors. It increases targeting and overcomes the possible alteration in cell surface epitopes associated with phage adsorption [38, 53]. Unfortunately, no precise official guidelines have been made yet, despite the fact that standardized methods of phage cocktails preparation have already existed [56, 57]. It has to be emphasise that detailed comprehensive genetic and phenotypic characterization of therapeutic phages has to be performed, to detect the unfavourable features such as toxic or harmful enzyme production or presence of lysogeny encoding genes [34]. Nowadays the new generation sequencing methods allow to study enormous number of phage genomes, however, the big part of phage genes are still described as hypothetical, putative proteins with predicted or unknown function. The deliberations about the advantages and limitations of phage application as antimicrobials in comparison to antibiotics, including pharmacodynamic (PD) and pharmacokinetic (PK) aspects have been discussed in detail elsewhere [58].

3. PHAGE-ASSOCIATED POLYSACCHARIDE DEGRADING ENZYMES

Polysaccharide-degrading enzymes, also referred to as polysaccharide depolymerases, are virion-associated proteins employed by phages to enzymatically degrade the capsular (alginate, hyaluronan, polysialic acid, amylovoran) or structural (LPS, PG) polysaccharides of their hosts, at the initial step of a tightly programmed phage infection process (Fig. **1**).

The ability to overcome these structures by enzymes with polysaccharide depolymerization activity allows some phages to infect encapsulated *E. coli* K1 and K95 serotypes [59, 60], *V. cholerae* O139 [61], *P. aeruginosa* , *P. putida* and *P. agglomerans* strains [62-64], specifically recognize the bacterial polysaccharide and then degrade it, which is essential to gain access to the appropriate receptors responsible for irreversible phage attachment to the bacterial cell. Others phages, including for instance phage P22 and phage 9NA, hydrolyse the outer LPS layer to provoke DNA ejection and consequently further progress of the infection process, without the need for using other additional receptors [65, 66]. Most phage depolymerases can occur in two forms: (i) as an integral component of a virion particle, usually in the form of small tailspikes or fibre proteins attached to the base plate, though they may also be located in other positions (e.g. within the internal head or in the viral membrane), and (ii) as a soluble protein generated during host lysis after phage maturation [67].

Depending on the mode of degradation of carbohydrate-containing polymers on the surface of bacterial cells, phage-associated enzymes fall into two main groups: hydrolases (EC 3.2.1.-) and lyases (EC 4.2.2.-) [68]. The first ones degrade either the peptidoglycan, capsular polysaccharides or the O-antigen side-chains of LPS. They catalyse cleavage of the glycosyl-oxygen bond in the glycosidic linkage by hydrolysis. Lyases, in turn, utilize the β-elimination mechanism to introduce a double bond between the C4 and C5 of the non-reducing uronic acid after cleavage of the glycosidic linkage between a monosaccharide and the C4 of uronic acid. Examples of such enzymes are alginate and hyaluronan lyases.

The polysaccharides surrounding the bacterial cells are an evolutionary achievement of prokaryotes. These conservative structures are important virulence factors protecting bacterial cells against both immune host defences and antibiotics. Numerous attempts of how to destroy these polysaccharides, are involved in practical novel approaches to combat pathogens. Phage-borne polysaccharide degrading enzymes alone or in combination with other agents represent a promising yet challenging antimicrobial therapy and useful diagnostic tools.

3.1. Peptidoglycan Hydrolases

Virion-associated peptidoglycan hydrolases (VAPGHs), analogous to endolysins discussed below, are phage-encoded lytic enzymes that specifically degrade PG, catalysing the breakdown of one of the four major bonds in it. According to their enzymatic activity they have been classified into at least four group: (i) lysozymes (e.g. gp5 from T4 phage); (ii) lytic transglycosylases (e.g. gp16 from T7 phage, protein P7 from PRD1); (iii) glucosaminidases and (iv) endopeptidases (e.g. protein P5 from phi6 phage, Tal2009 from Tuc2009 phage) [69-73]. It should be noted that in the light of the current International Union of Biochemistry and Molecular Biology (IUBMB) enzyme nomenclature [74] all the VAPGHs are hydrolases except for lytic transglycosylase. The latter cleaves the β-1,4-glycosidic bond based on an entirely different mode of action. To simplify, the term "PG hydrolases" for this whole group of enzymes will be used subsequently in this review. Unlike the endolysins employed by phages to release progeny particles from the bacterial cell at the end of their lytic cycle [75], the VAPGHs are involved in the initial stage of phage infection, prior to phage replication,

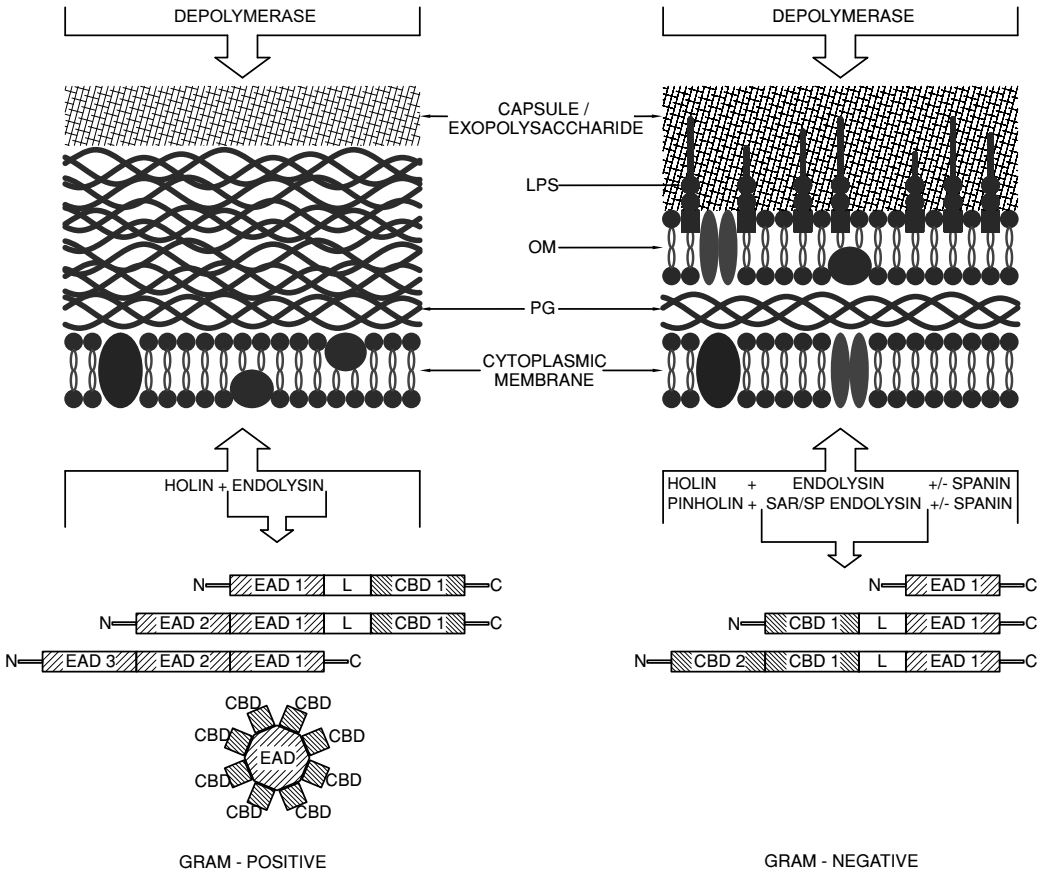

Fig. (1). Phage-derived proteins: location and organization system.

facilitating the penetration of the cell wall by the tail tube and promoting subsequent injection of the genome into the host cell [76]. In fact, the function of VAPGHs during the phage lytic cycle is ambiguous. These enzymes degrade the cell wall in order to reach the host cytoplasmic membrane during virus entry. Beyond this generally accepted principle, it has been reported that some VAPGHs, such as gp49 from *S. aureus* phage phi11, as well as gp16 from *E. coli* phage T7 or protein P7 of PRD1, are not essential for phage multiplicity [71, 73, 77]. However, their presence provides the stability of viral particles and enables infection under less optimal conditions (e.g. cells grown at low temperature or too high cell density). It is speculated that such proteins may succour the phage infection under conditions in which the PG layer possesses greater than average levels of cross-linking (for a recent review, see [78]). Likewise, the reduced lysozyme activity in the well-studied *E. coli*-infecting T4 phage was not associated with the failure of its capability of infecting a host cell, although this process was significantly delayed [79]. On the other hand, antibodies specific for the tail-associated lysine (Tal2009) encoded by *Lactococcus lactis* phage Tuc2009 decreased the phage ability to infect its host more than 100-fold [80].

The presence of the PG layer in the cell wall of both Gram-positive and Gram-negative bacteria appears to indicate widespread occurrence of virion-associated muralytic activities, as confirmed by zymogram analysis or by homology analysis of sequenced phage and prophage genomes. To date, the VAPGHs have been revealed in phages phiMR11 (gp61) [81], P68 (P17) [82], phiIPLA88 (HydH5) [83], K (P128) [84], phi11 (gp49) [77] infecting *S. aureus*, in phages sk1, r1t, c2, Tuc2009 infecting *Lactococcus lactis* [80, 85] as well as in phages specific to *Bacillus subtilis* such as phi29 (gp3) [85] and SP-β (CwlP) [86]. Besides the phages specific to Gram-positive bacteria, the PG hydrolases from *E. coli* phages such as T4, T5, T7, PRD1 [71, 85, 87-89], *Pseudomonas aeruginosa* (phiKZ, phiKMV) [90, 91], *Pseudomonas syringae* (phi6, phi13) [70, 92], *Salmonella* Typhimurium (P22 phage) [85] have also been identified. Given the sophisticated strategies of cell entry employed by these phages, the structural position of their VAPGHs seems to be the optimal location for contact with the PG layer of the host cell. It has been shown that the PG hydrolytic activity of aforementioned phages may be associated with individual proteins located: (i) at the tail tip (e.g. Tal2009 of Tuc2009 phage, gp61 of phage phiMR11); (ii) at the tail baseplate (e.g. gp5 of T4 phage, gp181 of phiKZ phage); (iii) within the internal head (e.g. gp4 of P22, gp16 of T7); (iv) in the viral membrane (e.g. P7 and P15 of PRD1); (v) in the nucleocapsid (e.g. P5 of phi 6) [70-72, 80, 81, 85, 88, 91]. Apart from proteins anchored to the virion, the same activity can also be found on catalytic domains included in other proteins [85].

The phenomenon called 'lysis from without' was first reported in 1940 [93, 94]. In this process, the rapid disruption of the bacterial cells occurs, without phage production, following too high number of some phages adsorbed onto their

surface. Similar process related to PG degradation has been well documented for endolysins in controlling Gram-positive bacterial infection (reviewed by Schmelcher *et al.*, [95]; Fischetti [13]; Pastagia *et al.*, [96]). From that time the VAPGHs have emerged as novel antimicrobial agents for pathogens eradication (both in medicine and in industry or biotechnological settings) [78]. Promising results regarding the antimicrobial activity of the VAPGHs against both Gram-positive bacteria and Gram-negative bacteria have already been proposed [70, 81-83, 91]. Recently, attention has also been paid to the enzybiotic potential of purified VAPGHs derived from phages excluded from applications in therapy due to their temperate nature [97]. Moreover, the unique properties of these enzymes, such as (i) high thermostability; (ii) a modular design and (iii) specificity, further actively support their enzybiotic applications. The high tolerance to heat and ionic strength make the VAPGHs attractive antimicrobials to be exploited in food technology, alone or in combination with other sanitization procedures. For example, they could be used as antimicrobial additives for controlling undesirable bacteria in foods that need to be heat treated, such as pasteurized milk or other dairy products. Rodriguez-Rubio and colleagues have demonstrated that the PG hydrolase HydH5 encoded by *S. aureus* phage phiPLA88 and its derivative fusions preventing the growth of *S. aureus* both in pasteurized and in raw (whole and skimmed) milk [98]. Due to that they could be considered as a promising agent to control *S. aureus* causing bovine mastitis. In turn, the modular organization of the VAPGHs enables scientists to design and construct functional chimeric proteins consisting of multiple domains of unique origin. Using a strategy of domain swapping or random mutagenesis can modify properties of enzymes related to solubility, thermostability, binding specificity and catalytic efficiency in order to obtain the optimal chimeric protein for specific applications. A study carried out by Rodriguez-Rubio *et al.* [99] have revealed that different recombinant fusion proteins between the HydH5 and the SH3b staphylococcal cell wall-binding domain of lysostaphin show a significant increase in lytic activity compared to the parental protein (HydH5) and lyse both *S. aureus* and *S. epidermidis*, including the methicillin-resistant strains (MRS). Additionally, the synergistic effect of these constructs and endolysin LysH5, encoded by the same phage, has been demonstrated. Also, Paul and co-workers [84] have been able to improve the staphylococcal lytic activity of TAME, encoded by phage K, via fusion with the lysostaphin SH3 domain, creating the chimeric protein P128, which was shown to have bactericidal activity against MRSA clinical isolates both *in vitro* and *in vivo*, using a rat nasal colonization model. Another example of a particular enzybiotic against *S. aureus* is a chimeric protein (P16-17) composed of the inferred N-terminal endopeptidase domain and the C-terminal cell wall targeting domain of phage P68 endolysin (Lys16) and the VAPGH (P17), respectively [100].

3.2. Endosialidases

The endosialidases, also termed endo-N-acetylneuraminidases (endoNs, EC 3.2.1.129, a group of glycosyl hydrolases) are found as specialized tailspike proteins of phages infecting the pathogenic bacterium *E. coli* K1 [101, 102]. Crucially, as we know to date, these are only enzymes specifically recognizing and hydrolysing an internal α-2,8-linkages in polysialic acid (polySia), being a linear carbohydrate polymer composed of N-acetylneuraminic acid units [103, 104]. PolySia is the central component of capsular polysaccharide of both aforementioned neuroinvasive *E. coli*

K1 and other bacterial pathogens such as *Neisseria meningitidis* serogroup B, *Moraxella nonliquefaciens*, *Pasteurella haemolytica* A2 and *Haemophilus influenzae* type b cause meningitis and septicaemia [105-108]. Due to its mimicry to the host structure, the polysaccharide in the capsular material allows the bacteria to avoid the host immune defence, which certainly enhances their pathogenicity [109]. Besides the fact that polySia is both a virulence factor and a protective antigen of neurotropic bacterial pathogens, it is also a component of normal human tissue [110, 111]. Almost 30 *E. coli* K1-specific phages, that contain enzyme with polySia-depolymerizing activity have been identified [112-120] and endoN genes as functional proteins from at least five phages (K1A, K1E, K1F, 63D, K1-5) have been cloned and expressed to date [119, 121-125]. All these phages have been classified as linear dsDNA viruses and morphologically most of them belong to the *Podoviridae* family, although representatives of *Myoviridae* [114] and *Siphoviridae* [116, 120] have also been reported. Apart from lytic phages, several temperate phages with endoN genes have been described [119, 126-131]. A comprehensive review concerning the structure and the biochemical properties of endoNs as well as the significance of these enzymes in medical and biochemical applications (briefly discussed below) has been published by Jakobsson and co-workers [132].

Although endoN alone is not sufficient to destroy the bacterial outer membrane and kill the bacteria, its unique ability to degrade bacterial capsular material can be applied to decrease pathogen virulence. Even treating the life-threatening systemic infections caused by neurotropic strains of encapsulated bacteria could be possible by processes that do not involve direct bacterial lysis, but rapid degradation of capsular polysaccharide only. The loss of K1 antigen, which is the primary virulence factor of these pathogens, essential for bacterial dissemination and survival within the host, sensitizes the modified phenotype of bacterial cells to either conventional antibiotics or a component of the host's immune system such as the bactericidal action of complement or phagocytosis by macrophages [133]. Therapeutic efficacy of the enzyme-mediated "capsule-stripping" phenomenon has been confirmed in an animal model. It has been shown that intraperitoneal administration of endoN E to infected neonatal rats in the initial stage of the infection's development selectively removes the capsular polysaccharide from the *E. coli* surface and interrupts the transit of bacterial cells from gut to brain via the blood circulation, preventing bacteraemia and death from systemic infection [133, 134]. Additionally, it was observed that the reduction of K1 antigen did not affect the viability of bacteria at the site of infection. Thus, the success of endoNs seems to be related to their ability to the attenuate *E. coli* K1 virulence by degradation of the polySia capsule, and consequently to restrict the inflammatory processes and tissue damage occurring in response to bacterial invasion. Thereby the endoNs, without help from membrane-disrupting agents or accessory proteins, can sig-

nificantly reduce the mortality and morbidity associated with *E. coli* systemic infection [135]. PolySia is found not only in a capsular material of some bacteria but being a post-translational modification of the neural cell adhesion molecules (NCAM) serving also as a modulator in the process of neuronal development [136, 137]. The unique ability of phage-borne endoNs to specifically degrade polySia, as well as the stability and activity even at physiological pH and ion conditions, has promoted their application in polySia research, covering neurobiology and oncology [138-141]. Furthermore, these enzymes can be utilized for artificial polySia-based hydrogel degradation and other derivatives at strictly-defined time points, serving as a scaffold biomaterial for engineered nerve grafts tissue [142]. Besides the versatile applications of active endoNs described above, these enzymes may also be applied in catalytically inactive variants, which can still recognize accurately and to bind to polySia substrate without its enzymatic degradation. Potentially, such engineered non-catalytic endoNs could be used as an equivalent of the antibody and could become an accurate tool applied both in immunohistological studies and in microbial diagnosis. As a detection reagent in identification of polySia-containing bacteria or eukaryotic cells, inactivated enzymes have many advantages in comparison to antibodies, such as simple and reproducible production based on recombinant techniques (no necessity of animals use), and the lack of cross-reactions with antibody-binding proteins located on tissue cells. Their widespread utility could also be supported by elimination of the additional difficulty involved with obtaining antibodies, in this particular case, related to the poor immunogenicity of polySia [143].

3.3. Endorhamnosidases

Endorhamnosidases (EC 3.2.1.-) are used by some phages for explicit recognition and depolymerization of repetitive carbohydrate structures of O-antigen in the LPS molecule. They catalyse the cleavage of the α–(1→3) O–glycosidic bond between L–rhamnose and D–galactose, yielding mainly octasaccharide fragments of two repeating units with rhamnose of the reducing end [144, 145]. They have been classified as glycoside hydrolase family 90 [146]. Their enzymatic activity is mediated by tailspikes of phages (e.g. P22, 9NA, ε15, ε34, Det7) specifically infecting *Salmonella* strains [66, 147-150] and other phages, e.g. *Shigella* phage Sf6 [151] or coliphage Ω8 [152, 153]. The reason, for which the phages recognize and hydrolyse the long O-antigen chains of the LPS, is not unequivocal. Enzymatic cleavage of this polysaccharide might facilitate access to the membrane and the appropriate receptor [150, 154]. On the other hand, Andres *et al.* [65, 66] have proved that the interaction of the tailspike protein of both the well-known short-tailed podovirus P22 and the long-tailed siphovirus 9NA with the LPS give a signal to modulate DNA egress from the phage capsid and start the infection process. Despite the differences in their tail structure, phages hydrolysing the O-antigen do not use the further secondary receptor (i.e. protein) on *Salmonella* surface for irreversible attachment. Also, it cannot be excluded that some unknown protein receptors are involved in the process. In addition to the role in the early event of the infection cycle, the endorhamnosidase activity associated with tailspikes may facilitate the release of phage progeny from sticking cellular debris generated during host lysis [65, 66].

3.4. Alginate Lyases

Alginate lyases, characterized as either mannuronate (EC 4.2.2.3) or guluronate lyases (EC 4.2.2.11), catalyse depolymerization of alginate, a linear copolymer of (1,4)-linked α-L-guluronic acid residues and its C5 epimer β-D-mannuronic acid, arranged in blocks of polymannuronate (M), polyguluronate (G) or heteropolymeric M/G random sequences [155]. Alginate can be formed by some bacteria including *Pseudomonas* and *Azotobacter* genera, as well as by brown seaweed [156, 157]. The bacterial polysaccharide, as opposed to alginate synthesized by algae, mainly consists of polymannuronate with O-acetyl groups at the C-2 and/or C-3 positions of mannuronate [158], making this compound less sensitive to the endogenous lyase degradation [159, 160]. Alginate is one of the three major *Pseudomonas* exopolysaccharides, and it is only produced by mucoid strains that establish chronic infections in patients with cystic fibrosis (CF). The ability to synthesize the alginate, the additionally a crucial component of mucoid biofilm structure, protects the pathogen from the host defence mechanisms and antibacterial agents. It is known that organisms living in biofilm are 10-1000-fold more resistance to antibiotics than corresponding planktonic cells. On the other hand, this exopolysaccharide can also constitute an attractive target for alternative therapies. The concept of using phage alginate-degrading enzymes as antimicrobials to prevent and control biofilm-associated infections, including *Pseudomonas* lung infections in CF patients, was initiated in the late 1960s. It-still remains one of the most important purposes of research on them. These enzymes allow the phages to invade the bacterial cells entangled in the polysaccharide backbone and kill them mainly by dissolution or disruption of the EPS layer in the biofilm structure. Moreover, the released bacterial cells, following the dispersion of the EPS from the biofilm, can again be accessible for antimicrobials or host immune system components [161-163]. As shown in one of the preliminary reports, depolymerases purified from crude lysates produced by the propagation of *P. aeruginosa* specific phages were responsible for reduction in the viscosity of the extracellular slime polysaccharides and increased levels of hexosamines, hexoses, and reducing substances [164]. Although the exact structure of this enzyme has not been elucidated, its enzymatic activity, located in an 180 kDa component, indicates the adsorption apparatus composed of six drop-like tail spikes [62, 165]. In turn, Hanlon *et al.* [166] have showed that *P. aeruginosa* phage penetration through purified CF alginate can done easier following the decrease of viscosity and the molecular weight of alginate brought about by enzymatic degradation. More recently, several *Pseudomonas* phages, which are able to degrade four structurally different pseudomonal alginates, have also been identified [167]. The majority of phage alginases studied to date have molecular masses in the range from 30 to 42 kDa and optimal pH for their endolytic activity between 7.5 and 8.5 [155, 167]. Considering the anti-biofilm effects of alginate lyases, one also cannot exclude enzyme-mediated changes in cell physiology. Recently, a novel potential mechanism underlying anti-biofilm effects of alginate lyases has been sug-

gested, in which the synergistic action of the antibiotic and enzyme without catalytic activity, allows for removing the infection [168]. Researchers suggest that in response to the intact protein treatment (e.g. BSA), the bacterial cells secrete proteases that destabilize the crucial component of the biofilm matrix and contribute to biofilm disruption. Subsequently changes in the metabolism of bacterial cell induced by the released amino acids may lead to improving its susceptibility to antibiotics.

3.5. Hyaluronate Lyases

Hyaluronan (hyaluronic acid, HA), a polysaccharide composed of a linear repetition of β-1-4 linked N-acetylglucosamine and D-glucuronic acid subunits, is a sole or dominant component of the capsular material of certain bacterial strains as well as an important part of the extracellular matrix of body tissues [169, 170]. The enzymatic activity of this polysaccharide has been associated with a variety of organisms, including mammals, insects, leeches, bacteria and phages [171]. In contrast to the hyaluronidases of eukaryotic origin that act hydrolytically, breaking the glycosidic β-1,4 bound, both bacterial and phage-encoded enzymes (better known as hyaluronate lyases (HyaLs), EC 4.2.2.1) cut the same linkage via a β-elimination mechanism [172]. Bacterial and phage-derived lyases have very little amino acid sequence homology, and they also differ in structure and substrate specificity. The former are monomeric proteins with their N-terminal domain being the catalytic domain [173] while the latter is oligomeric [174] with the active site located at the C-terminal domain [175]. Moreover, unlike most bacterial lyases, acting non-specifically on both hyaluronate and chondroitin sulfates, the phage enzymes recognize HA as its only substrate [176]. Another unique feature of phage HyaLs is their molecular mass ranging from 36 to 40 kDa, the lowest among these enzymes of different origin identified so far [177]. All identified phage HyaLs have been classified in the PL16 family within the Carbohydrate Active enZYmes (CAZy) database [178] based on recognizable sequence homologies. Contrarily, the streptococcal proteins belong to a different polysaccharide lyase group, the PL8 family. To date, at least several phage HyaLs have been identified which demonstrate activity against streptococci including hylP from phages H4489A [176, 179], HylP1 (phage 370.1) [174], HylP2 (phage 10403) [177, 180], HylP3 [181] and SEQ2045 [182]. They are mainly found in phages invading two species of group A streptococci: *S. pyogenes* and *S. equi*. Most genes encoding these enzymes are carried within the prophages, that are integrated into bacterial chromosomes. A possible function of the phage HyaLs involves the local degradation of the HA layer and capsule viscosity reduction, after attachment of the phage particle to the cell, so that the phages can gain access to the appropriate receptors hidden inside and infect the encapsulated cells [177, 179, 183]. Although the phage HyaLs may be tightly bound to, or constitute an integral component of, the virion, most of the HyaLs produced by infected strains appear to be free and not associated with phage particles [184]. This proves that after induction of the lytic cycle, temperate phages may multiply and produce of progeny virions and free enzyme. Thus, it is not inconceivable that beyond the direct role of HyaLs associated with overcoming the capsule, they may also help in spreading the infecting bacteria from their initial site of infection, in essence acting as a virulence factor for the host bacterium. In addition, due to the possibility of the phage HyaLs to degrade the HA of human connective tissue, these enzymes may also promote spread of phage gene encoding erythrogenic toxin, that causes the visible rash in scarlet fever [185]. On the other hand, owing to the defined specificity of these enzymes, they could be applied in structural studies of connective tissue glycosaminoglycans.

4. PHAGE-DERIVED PROTEINS INVOLVED IN PROGENY RELEASE

Bacterial viruses possess diverse strategies to release of progeny from infected bacteria. They achieve this goal by: (i) constant phage budding without or with very gradual killing of the host; (ii) extrusion, a process that leaves the cells fully viable; (iii) PG synthesis blocking "slow" lysis; or (iv) an abrupt disintegration of the host achieved by phage lytic enzymes. The budding (gemmation) process is the distinguishing feature for pleomorphic phages, represented by the non-capsid *Plasmaviridae* family propagating on *Mollicutes* lacking the cell wall structure. During the budding action, the phage particles become covered by bacterial lipoprotein membrane and the releasing pattern depends on the type of the bacterial envelope. To our knowledge examples of phages able to liberate virions by gemmation are enveloped, quasi-spherical, dsDNA and *Acholeplasma*-specific phages: L2 (MV-L2) or L172 [186-192]. A budding-like mechanism, but in contrast to typical gemmation, terminating with the slow death of the host, is utilized by short-tailed dsDNA, phages L3, SpV3 and *ai* isolated from *Acholeplasma laidlawii*, *Spiroplasma mirum* and *Spiroplasma citri*, respectively. Although these phages can produce clear plaques, their progeny is not released by typical cell lysis. Mature L3, SpV3 and *ai* viruses accumulate in infected cells and are released continuously over many hours as extracellular membrane vesicles enclose one or more progeny virions. The escape of large quantities of phage particles, in a short period, leads to destruction of host cell integrity. Furthermore, the vesicles containing viral particles may finally break down and liberate non-enveloped virions [186, 188, 193]. The second type of release mechanism presented by extrusion is complicated, closely associated with the assembly process and characteristic for ssDNA filamentous phages [194]. The intracellular DNA-binding proteins produced by these viruses bind to each copy of the replicating phage single-stranded DNA. At this time, structural and morphogenetic phage proteins localize in the membrane of the infected cell, forming a channel and coat, where the genetic information is extruded creating the mature virion. [195-198]. The third strategy, based on PG biosynthesis inhibition, is exploited by small-genome ssDNA and RNA phages. These viruses encode low-molecular-mass hydrophobic proteins that act in a manner reminiscent of cell wall antibiotics blocking the murein synthesis pathway, which is sufficient to trigger lysis of the host. The mechanism employing a single lysis protein has been well studied in the example of protein E encoded by *E. coli* phage phi X174 (ssDNA). Protein E functions as an inhibitor of phospho-MurNAc-pentapeptide translocase (MraY) which is involved in the biosynthesis of bacterial mureine [199-201]. Other examples are ssRNA

coliphages Qβ and MS2 encoding protein A_2, that blocks cell wall biosynthesis by inhibiting UDP-N-acetylglucosamine enolpyruvyl transferase (MurA), an enzyme catalysing the first stage in PG synthesis [202] and protein L lacking muralytic activity with an unknown mechanism of induced lysis, respectively [203-206]. The fourth mechanism, involving the use of lytic enzymes for fast destruction of the host envelopes and progeny release, greatly predominates among all described phages and is of particular importance for practical reasons. The mechanism of lysis in this scenario is employed by tailed dsDNA bacteriophages which constitute the vast majority of viruses replicating in bacteria, meaning more than 90% of all phages with completely sequenced genomes and published in NCBI databases. During the release process, progeny virions have several barriers, represented by cell envelopes, to overcome. These are in sequence: the inner cell membrane (IM), the PG layer, and additionally an outer cell membrane (OM) in the case of Gram-negative bacteria (Fig. **1**). The dsDNA bacteriophages encode at least two enzymes, holin, and endolysin, to cause destruction of the inner membrane and murein, respectively. The third type of protein, spanin, responsible for crossing the outer membrane, is characteristic of highly specialized, Gram-negative specific phages. Holins determine the exact time of bacterial lysis by controlling the access of phage muramidases to the PG layer. In view of this, holins are considered as the simplest biological timing system [205, 207, 208]. These proteins are very diverse, but they also share common features: (i) they are encoded by short genes, usually up to 110 codons, (ii) their genes are mostly adjacent to the endolysin gene creating the so-called two-component lysis cassette; (iii) they possess hydrophilic, positively charged C-terminal domain; and (iv) they consist of a minimum of one and a maximum of three hydrophobic transmembrane domains (TMDs) [209-212]. Depending on the number of TMDs, holins can be divided into three classes. Proteins grouped in the first class possess three TMDs, and examples of these are HolSMP protein, encoded by *Streptococcus suis* phage SMP, P68 hol15 derived from *Staphylococcus aureus* phage P68 [213] and S protein encoded by *Enterobacteria* phage λ [214]. Class II holins, characterized by two TMDs, include pinholin S encoded by coliphage 21 (for pinholins see below) [215] and Hol3626 protein from *Clostridium perfringens* bacteriophage Φ3626 [216]. Class III holins, with just one TMD, are produced by coliphage T4 (T protein) and *Clostridium perfringens* bacteriophages φCP39-O (39-O_gp28) and φCP26F (phi26F_gp23) [211, 213, 217]. The most likely and primary principle of the enzymes' initiation of the phage lytic machinery has been described by Krupovic [218] and Wang [208]. Phage holins are able to accumulate in the inner membrane until achieving the critical concentration and may create: (i) large channels forming a passage for endolysin to reach the murein layer, or (ii) small pores essential for endolysin activation [215, 219-222]. Large channel forming holins change conformation leading to oligomerization and finally to gap formation in the inner membrane. The holins, acting in this manner, form a nonspecific tunnel to transport endolysin or an endolysin complex in a maximum size of 500 kDa [208, 218] Small pore-forming holins, inappropriately named pinholins, cooperate with endolysins by formation of symmetric protein heptamers with the central channel of about 15 Å in diameter. This pore is too narrow to transfer endolysin molecule but broad enough to allow movement of ions, a process crucial for cell membrane depolarization. Local depolarization is prerequisite for SAR (signal arrest release) or SP (signal peptide) endolysin activation [215, 219, 220]. Endolysins, also termed lysins are phage enzymes employed in fast enzymatic degradation of PG. Among certain endolysins signal arrest release (SAR) or simple signal peptide (SP) domains mediate the transport utilizing bacterial *sec* system and associate an inactive endolysin with the inner cell membrane, finally participating in lysin activation [223-225]. Endolysins with the SAR element, as described in the example of coliphage 21, are synthesized, transported and anchored to the inner membrane in inactive form [222, 226]. Based on the type of holin (canonical or pinholin) and type of endolysins (with or without the SAR/SP signal), lysis systems are divided into two general types: the canonical holin-endolysin system and the pinholin-SAR/SP endolysin lysis system (Fig. **1**).

Based on endolysins origin, these enzymes can be divided into those derived from Gram-positive specific or Gram-negative specific phages. Another classification relies on cleavage site of endolysins and divides these proteins into four general groups: (i) glycosidases (lysozyme and *N*-acetyl-β-D-glucosaminidase), cleaving the polysaccharide backbone of PG; (ii) lytic transglycosylases destroying linkages between N-acetylmuramyl and N-acetylglucosaminyl residues of PG but other than muramidase, forming the N-acetyl-1,6-anhydro-muramyl moiety residue; (iii) N-acetylmuramoyl-L-alanine amidases cleaving the amide bond between *N*-acetylmuramic acid and the L-alanine of the stem peptide; and (iv) endopeptidases capable of cutting either the stem peptide or cross bridges of PG (L-alanoyl-D-glutamate endopeptidases, D-glutamyl-m-DAP endopeptidases, interpeptide bridge-specific endopeptidases, D-alanoyl-m-DAP endopeptidases, glycylglycine endopeptidases, D-alanoyl-glycine endopeptidases, D-glutamyl-L-lysine endopeptidases, D-alanoyl-L-alanine endopeptidases) [58, 95, 219, 227-229]. Another, simpler distribution of endolysins is based on the gene structure and organization. It distinguishes (Fig. **1**): (i) globular proteins with a single enzymatically active domain (EAD) prevalent among the endolysins encoded by Gram-negative specific phages; and (ii) modular enzymes composed of an enzymatic domain and cell wall-binding domain (CBD), mostly found among Gram-positive specific phages. The CBD function include the specific recognition of the moieties in the targeted cell wall structure with high-affinity binding and endolysin immobilization on bacterial debris to protect uninfected host cells against enzymatic degradation leading to lysis. Such risk of cell destruction by endolysins does not exist among Gram-negative hosts, because of the presence of an extra protective outer layer membrane. Most of the studied modular endolysins are composed of two clearly separated functional domains – N-terminal catalytic and C-terminal binding domains – but their location and number may vary. Endolysin genes can have up to three domains in different combinations of orientation. The composition of modular lysin architecture may consist of various configurations: one or more EAD domains from the aforementioned, used for PG bond hydrolysis and the CBD element [13, 228, 230]. The vast majority of endolysins have a molecular mass in the

range of 25-40 kDa [13], There is one exception to date, protein of 114 kDa, a multimeric PlyC endolysin of *Streptococcus* phage. This large mass is a consequence of complex structure, which consists of one substantial catalytic domain named PlyCA and eight chains responsible for binding to the bacterial cell wall (PlyCB). [231]. Diversity in endolysin domain organization, with examples provided, has been described in detail by several authors [228, 229, 232]. As soon as the barrier set up by the bacterial cell wall is devastated by endolysins, phage progeny proliferating in Gram-positive bacteria are free to leave and attack other susceptible host cells. However, the matter is not so simple in the case of Gram-negative bacteria due to the presence of another barrier (OM) that must be crossed. Although the destruction of IM and PG layer leads to tearing the OM under favourable external conditions (the presence of millimolar concentrations of divalent cations), it can withstand the pressure and keep progeny inside or at least extend the time of their release [233-235]. Therefore, Gram-negative specific phages had to develop an additional tool, namely spanins. The spanin encoding gene is typically located near the endolysin and holin genes, creating a three-component lysis cassette. Examples of phages containing holin, endolysin and spanin genes in canonical order are well known in *E. coli* phages λ and P2 as well as *Pseudomonas* phage φKMV. However canonical order of lysis genes, is not the universal rule and Catalao and co-authors have described a large variety of lysis genes and their arrangement in phages genomes [219]. The best-described spanin mode of action is for λ phage. This phage possesses two separately synthesized proteins: (i) Rz named i-spanin, a type II integral membrane protein with one N-terminal transmembrane domain and a large alpha-helical periplasmic domain on the C-terminus; and (ii) Rz1 named o-spanin, a small, proline-rich, outer membrane lipoprotein with a signal peptidase II cleavage site [219, 235, 236].

The active complex of Rz and Rz1 (an i-spanin/o-spanin complex) extends across the entire width of the periplasm and act as the physical connection between the inner and outer membranes. These chains are immobilized by the PG layer, making murein a negative regulator of spanin function [235, 237]. Removal of PG by endolysins allows for lateral oligomerization and conformational changes of spanins, consequently leading to OM destabilization [235, 238]. Spanin can also occur as a single protein (unimolecular, u-spanin) acting individually. Functional homologs of Rz/Rz1 complex, but existing and acting as a single protein, have also been identified in some phages as exemplified gp11 spanin encoded by *E. coli* phage. However, it is believed that the still very poorly understood mode of action of all spanins looks similar to that described above [235, 236]. With all of the mentioned proteins involved in the phage progeny release strategy, some are particularly interesting and significant due to their potential usefulness. Among proteins with therapeutic potential the the aforementioned murein synthesis inhibitors seems to be quite promising. Yu *et al.* [239] have demonstrated that a 91-amino acid residue of protein E, referred to above, which plays a significant role in host cell lysis in φX174 phage propagation, can be used for generation of so-called bacterial ghosts. These empty bacterial bodies lacking cytoplasm and nucleic acids exhibit excellent immunogenicity and provide effective inducible immunoprotection, and therefore can be used directly as vaccines [239]. Analogously, proteins A_2 and L encoded by *E. coli* specific phages Qβ and MS2 can be potentially applied to inactivated bacteria preparation. Phage holins can be utilized to create empty bacterial ghosts from Gram-positive strains. Capacity, to form translucent bacterial ghosts has been reported for protein HolNU3-1 from *Staphylococcus aureus* (MRSA) NU3-1 [240]. However, the greatest and also confirmed practical significance has been demonstrated by phage endolysins. Recently, many reviews concerning endolysins application as antimicrobial agents with high application potential in the fields of medicine, food safety, veterinary, cosmetic and chemical industry, agriculture and biotechnology have been published [58, 95, 96, 229, 241-243]. Due to confirming the efficacy of phage lysins, the use of endolysins in the treatment of bacterial infections induced in laboratory animals deserves particular attention [244-253]. Although the first studies on endolysin bactericidal efficacy have been confirmed only for Gram-positive strains, it is already known that after modification such as mixture or fusion with peptides with OM-disrupting properties, these enzymes can be successfully used to fight Gram-negative bacteria [220]. Practical use is also attributed to endolysin-derived CBDs as well as receptor binding protein (RBP), an element of tail fibres or spikes, both combined with particular dye or magnetic beads, which due to their high specificity are successfully applied in diagnostics and foodborne pathogen detection [15, 22, 95, 254-256]. To summarize, depending on the strategy chosen by the lytic phage to release its progeny, the host remains alive, or it is slowly or rapidly lysed. Enzymes involved in cell lysis, such as murein inhibitors, holins, endolysins and spanins, by virtue of the fact that they are encoded by natural bacterial enemies, may serve as new tools for fighting bacterial infections.

5. CONCLUSIONS

Nowadays, fast-developing molecular biology techniques and genetic analysis methods allow the development of novel and safe antimicrobial agents based on natural bacterial parasites. There is much evidence of efficient application of environmental lytic and filamentous bacteriophages in treatment of bacterial infection, as well as some new reports on the use of temperate phages as inhibitors of pathogen virulence. Phages as antimicrobial tools, regardless of the life cycle nature and origin (lytic, non-lytic, temperate), prior to any clinical trials, must undergo a detailed analysis in terms of biology and genetic features to avoid unfavourable consequences as increase in bacterial virulence or/and an adaptive response to phage infection. The attractive solution to utilize safety and well-characterized phage product is the construction of genetically engineered phages, which give the opportunity to create all required features. Synthetic biology enables one to produce and design phage-based proteins instead of whole phage particles showing a broader antibacterial spectrum, better tissue penetration, lower immunogenicity and low probability of bacterial resistance.

The capabilities of modern synthetic biology and molecular engineering allow for relatively straightforward manufacturing of phage products such as recombinant peptides or synthetic analogs. Production and purification of aforementioned proteins on a large scale, are possible due to the well-

developed pharmaceutical technologies. The biggest advantage of alternative antibacterials, as phages or phage-based products, is the possibility of multi-agent treatment, including antibiotics, phages, and phage proteins. The idea, to combine biological-chemical treatment may help in phage and antibiotic resistance development prevention. The deepening knowledge of phage genetics and biology can lead to successful application of phages and phage-derived particles in therapy, and has already been existing happening in diagnostics, bacterial detection, preservation of food products, animal breeding and agriculture.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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