Taking aim on bacterial pathogens: from phage therapy to enzybiotics
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The bactericidal activity of bacteriophages has been used to treat human infections for years as an alternative or a complement to antibiotic therapy. Nowadays, endolysins (phage-encoded enzymes that break down bacterial peptidoglycan at the terminal stage of the phage reproduction cycle) have been used successfully to control antibiotic-resistant pathogenic bacteria in animal models. Their cell wall binding domains target the enzymes to their substrate, and their corresponding catalytic domains are able to cleave bonds in the peptidoglycan network. Recent research has not only revealed the surprising rich structural catalytic diversity of these murein hydrolases but has also yielded insights into their modular organization, their three-dimensional structures, and their mechanism of recognition of bacterial cell wall. These results allow endolysins to be considered as effective antimicrobials with potentially important applications in medicine and biotechnology.

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Current Opinion in Microbiology 2007, 10:1–12

This review comes from a themed issue on Antimicrobials
Edited by Steve Brickner and Shahriar Mobashery

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DOI 10.1016/j.mib.2007.08.002

Introduction
Infectious disease experts have warned that there is now a compelling need to develop totally new classes of antibacterial agents, ones that cannot be thwarted by the same gene products that render bacteria resistant to antibiotics. Phage therapy represents such a ‘new’ class. Phages (bacteriophages) are viruses that invade bacterial cells; they are ubiquitous, obligate parasites that are highly specific to their host. Phages have been proposed as natural antimicrobial agents to fight bacterial infections in humans, in animals, or in crops of agricultural importance as well as for hygiene measures in food production facilities and hospitals. Although it is unlikely that phages will ever replace antibiotics, they may be useful when no effective antibiotics are available or in conjunction with antibiotics for better treatment of disease. Since the safe and controlled use of phage therapy will require detailed information on the properties and behavior of specific phage–bacterium systems, both in vitro and especially in vivo, more recently different types of purified bacteriophage lysins have been evaluated as anti-infective agents.

There are excellent reviews that, in recent years, have dealt with the different characteristics of phages and their applications, both as entire virions or some of their products [1,2]. Here, we will just outline the key points of phage therapy and focus on the recent work of endolysins, the phage-encoded murein hydrolases, their crystal structures, and their therapeutic potential to control bacterial pathogens.

Phage therapy, a history of ups and downs

The phage therapy history starts with the history of phage discovery, which for decades has been a matter of extensive debates and controversies over priority claims [1,3–5]. Briefly, Ernest Hankin first reported in 1896 the presence of an antibacterial activity against Vibrio cholerae and two years later, Nikolay Fyodorovich Gamaleya observed a similar phenomenon while working with Bacillus subtilis. In 1915, Frederick Twort re-introduced the subject advancing the hypothesis that such antibacterial activity could be due to a virus. Since Twort did not pursue his discovery, phages were ‘officially’ discovered in 1917 by Félix d’Hérelle. He proposed that this phenomenon was caused by a virus capable of parasitizing bacteria and named these viruses ‘bacteriophages’, a word that is derived from the fusion of ‘bacteria’ and ‘phagein’ (to eat in Greek).

Not long after his discovery, d’Hérelle in 1919 used phages to treat dysentery; this was the first attempt to use them therapeutically. However, the first reported application of phages to treat infectious diseases in humans came in 1921 from Richard Bruynoghe and Joseph Maisin, who used bacteriophages to treat a staphylococcal skin disease. d’Hérelle and others continued the studies on phage therapy and in addition, several companies started in the 1930s the commercialization of phages against various bacterial pathogens. At this time, d’Hérelle established phage therapy centers in several countries, including the US, France, and Georgia [6]. During World War II the German and Soviet armies used
phages against dysentery, and the US army conducted classified research on it. Moreover, some practitioners used phages as therapeutic agents in the West, from the 1920s to the early 1950s, which was considered as the ‘historic era’ for phage therapy. However, phage therapy was generally abandoned soon after the introduction of antibiotics in the 1940s and thus, from the 1950s to the 1980s few data were published on this subject. Therefore, research regarding the therapeutic use of phages has been somewhat neglected in the West until the past two decades, when the increasing incidence of antibiotic-resistant bacteria awoke a renewed interest in phage therapy. Nevertheless, antibacterial phage therapy still presents a number of limitations that hamper its application as an antibiotic alternative [3,7–9]. The main drawbacks can be summarized as follows: (i) Phages tend to have a relatively narrow host range and one should administer only those phages strongly lytic for the bacterial strain infecting the given patient, which has to be identified before the administration of phage therapy; (ii) phages may not always remain lytic under the physiological conditions and bacteria can become resistant to phages after infection; (iii) phage preparations should be free of bacteria and their toxic components to meet strict clinical safety requirements, but sterilization of phage preparations could inactivate the phages; (iv) phages can be inactivated by a neutralizing antibody, and there is some risk of promoting allergic reactions to them; (v) the pharmacokinetics of phage treatments are more complicated than those of chemical drugs because of their self-replicating nature; (vi) phages might endow bacteria with toxic or antibiotic resistance genes. To overcome these concerns several experimental approaches aiming to use purified virions and phage lysins as anti-infective agents have been evaluated recently [10].

**Applications of entire virions**

Since the discovery of phages, they have been used in a variety of basic and applied research fields. A classical application of phages has been in diagnostics to identify and classify bacterial species and strains. As knowledge of phage structure and function increased, more applications of phages have been developed for the detection of pathogens. The dairy industry is far ahead of other industries in the exploitation of phages, as hundreds or even thousands of lactic acid bacteria infecting phages have been characterized around the world [11]. Interest has also grown in environmental application of phages, such as agriculture, aquaculture, and wastewater treatment. Polysaccharide-specific phages may prove very useful to treat encapsulated pathogenic bacteria. One area that shows the greatest potential for phage therapy is the treatment of persistent infections that are mediated through biofilm development and are difficult to treat with other antimicrobial agents. Such biofilms are widespread, pervasive, and often colonize indwelling medical devices, such as catheters and prosthetic heart valves. Furthermore, biofilm formation is a crucial factor in the pathogenesis of many bacteria, particularly those that are involved in persistent long-term infections. In the absence of effective strategies to deal with these infections, phage therapy has been suggested as a possible therapeutic option. As exopolysaccharide production in bacteria is typically involved in biofilm formation and acts as a barrier to the penetration of therapeutic agents, phages that can depolymerize these substances and/or kill the bacteria may potentially be useful for biofilm control. Some phages enter their chosen host via a specific enzyme that makes a hole in the outer coat of bacteria. An interesting case is the coliphage K1-5 that carries two capsule-specific enzymatic tail proteins, an endosialidase and a lyase, being capable of infecting bacterial strains with two different outer coats [12]. The reported engineering of dispersin B from T7 phage, a β-1,6-N-acetyl-d-glucosaminidase, that hydrolyzes the exopolysaccharide needed for biofilm formation and integrity in *Staphylococcus* and *Escherichia coli*, including *E. coli* K-12, as well as clinical isolates, represents a further advance [13–15]. This modified T7 phage reduced bacterial biofilm cell counts up to 100 000-fold better than that of non-enzymatic phage. Hydrolytic cleavage of the polysaccharide barrier of encapsulated pathogens can also be catalyzed by phage-encoded glycosidases themselves, as it has been demonstrated by the use of a phage-encoded endosialidase (endoE) as an antibacterial to treat infections caused by *E. coli* K1 strains [14].
To date, treatments for humans have been slow in reaching the market. This is partly because of regulatory hurdles; developing a new disease treatment and then showing that it is both safe and effective in humans takes years and costs millions of dollars. Thus, much of the initial work has been in food safety and animal health. But phage applications for human health show promise, especially for infections of the skin, inner ear, and other areas where phages can be applied directly and are less likely to be cleared by the immune system. Biodegradable patches containing a polymer matrix impregnated with a mixture of phages active against several Gram-positive and Gram-negative pathogens have been already used to treat patients with chronic skin infections refractory to many antibiotics [15]. Furthermore, Exponential Biotherapies appears to be the only company among a small field of competitors to have completed a clinical trial, a phase I study conducted in Europe, of a phage treatment for vancomycin-resistant Enterococcus faecium.

**Murein hydrolases**

Phages have developed two basic ways to release their progeny from bacterial cells [16]. Filamentous phages are continuously extruded from bacterial cells without killing them, whereas non-filamentous phages induce lysis of the host cell. Phage lytic enzymes are highly evolved murein hydrolases to quickly destroy the cell wall of the host bacterium to release the progeny. Lysis is the result of abrupt damage to the bacterial cell wall by means of specific proteins and can be accomplished in two different ways: (i) inhibition of peptidoglycan (PG) synthesis by a single protein or (ii) enzymatic cleavage of PG by lyisin(s) or holing–lysin systems.

Lysins (also termed endolysins) are enzymes encoded by phage genomes, which are produced during the late phase in the lytic cycle to degrade the bacterial cell wall, facilitating the release of virions [17]. Lysins must be distinguished from other phage-encoded lytic enzymes, which are an integral component of the virion tail and that locally digest the cell wall to facilitate the injection of the phage genome into the host cell. This kind of murein hydrolases appears to be widespread in the virions of phages infecting Gram-positive or Gram-negative bacteria. Where known, the PG lytic activity associated with tailed phage virions resides with the tail structure or with an internal head protein that forms part of the extensive tail. In other cases, the lack of genetic and structural studies and the proteolytic processing of structural proteins of some of these phages make difficult the complete identification of the protein with enzymatic activity [18]. A classical example of a well-studied process is the coliphage T4 where the tail tube uses a lysozyme inserted into a baseplate protein, at the tip of the tube [19].

Many lysins are produced as a single polypeptide but contain two functional domains, a catalytic domain, which cleaves PG bonds, and a cell wall binding module (CWBM), which may bind a species-specific carbohydrate epitope in the cell wall (see below). However, it has been shown recently that PlyC, the lysis from C1 streptococcal phage, is a multimeric enzyme composed of eight cell wall binding subunits for each catalytic subunit [20*].

Lysins can be classified according to their catalytic activity as N-acetylmuramidases (lysozymes or muramidases), endo-β-N-acetylglucosaminidases (glucosaminidases), N-acetylglucosaminyl-L-alanine amidases (NAM-amidases), endopeptidases, and lytic transglycosylases. Glucosaminidases, lysozymes, and lytic transglycosylases act on the sugar moiety (glycosidases), whereas endopeptidases cleave the peptide cross-bridge and NAM-amidases hydrolyze the amide bond connecting the sugar and peptide constituents of PG (see Glossary and Figure 1). Endopeptidases can also be subclassified as L-alanoyl-D-glutamyl endopeptidases, inter-peptide bridge-specific endopeptidases, and D-glutamyl-meso-DAP endopeptidases, though this latter activity has not been identified in a phage endolysin so far [17,21].

Remarkably, the lysins usually do not have a signal peptide to translocate them to the periplasmic space to access the PG and therefore phages encode small hydrophobic proteins termed holins, which export lysins across the bacterial inner membrane [16,17]. Nevertheless, there are also some examples of lysins that contain secretion signal and use the typical host sec system to reach the PG. Some lysins that are exported by the host sec system contain N-terminal signal sequences that function as a type II signal anchor or uncleaved signal peptide. This signal sequence has been designated SAR (signal arrest release) sequence because of the ability of the protein to escape from the membrane, leading to release into the periplasm [22*]. In this sense, it has been recently demonstrated that the eventual lysis of cells infected by coliphage N4 is due to the phage-encoded murein hydrolase, gp61, which contains an uncleaved N-terminal signal sequence [23*]. Interestingly, this lysis localizes to the inner membrane and could define a new family of muramidases. Finally, it is worth mentioning that some phages encode lysins without a known signal peptide but do not encode the corresponding holin and therefore these enzymes appear to be secreted by alternative mechanisms.

Lysins usually possess only one type of hydrolytic activity, but at least four enzymes harboring two independent lytic activities have also been reported so far in phages B30 and NCTC 11261 from Streptococcus agalactiae (endopeptidase and lysozyme), phage φ11 from Staphylococcus aureus (endopeptidase and NAM-amidase), and the prophage φWMY from Staphylococcus warneri M (endopeptidase and NAM-amidase) [17,24].Remarkably, the T7 lysis (an enzyme improperly named as “T7
lysozyme’ because it is a NAM-amidase and not a muramidase) also represents a particular case of a bifunctional protein since, in addition to the NAM-amidase activity, this enzyme plays a transcriptional regulatory role by binding to the T7 RNA polymerase and inhibiting transcription. Finally, although the bactericidal function of lysins is mainly ascribed to their hydrolytic activity on the cell wall, some lysins are capable of killing bacteria by means of a completely different mechanism. These lysins contain sequences in their C-terminus similar to those of cationic antimicrobial peptides, which promote membrane disruption, providing another interesting example of bifunctional antibacterial activity [16].

Purified lysins usually display a narrow spectrum of lytic activity, which is often restricted to the host bacterial species infected by the phage or at most to the host bacterial genus. However, there are some examples of lysins showing cell wall lytic activities that considerably exceed the range of bacteria infected by the phage from which they were derived [16].

Bacteria have also developed a set of chromosomal-encoded murein hydrolases that specifically cleave covalent bonds of the cell wall. There are some of these hydrolytic enzymes that, eventually, cause the lysis and death of the cell and, in this case, are named autolysins.
Phage lysins and host-encoded murein hydrolases share equivalent catalytic activities, and both are able to hydrolyze the PG, but their physiological roles are different. Bacterial murein hydrolases are not only involved in different processes, for example, cell wall remodeling, cell division, or transformation but may also play a fundamental role as virulence factors. In this sense, it is worth to mention that there are other bacterial enzymes that also hydrolyze or modify the cell wall components without producing a cleavage of PG chains, for example, phosphorylcholine esterases [26] and N-acetylglucosamine deacetylases [27]. These enzymes must also be taken into account since they can modify the activity of murein hydrolases.

There are many data supporting the hypothesis that host and phage murein hydrolases share common ancestry and, moreover, in some organisms it is evident that the proteins have co-evolved by interchanging their functional domains [25].

The original finding that lysins, when added to the cultures in their purified form, render Gram-positive bacteria susceptible to osmotic lysis and kill them within seconds, led researchers to consider these enzymes as putative antimicrobial agents. It is now well established that lysins, when added to sensitive bacteria in the absence of bacteriophage, are able to irreversibly damage the cell wall producing the lysis from without.

**Three-dimensional structure of endolysins**

The three-dimensional crystal structures of only a few endolysins have been reported and, in most of the cases, only the catalytic module was determined. The crystal structure of the modular pneumococcal phage Cpl-1 lysozyme [28] revealed for the first time the complete structure of a modular endolysin (Figure 2). Cpl-1 belongs to the CBP (Choline-Binding Proteins) family of proteins, with members in pneumococcus, its bacteriophages, and other related bacteria. All CBP proteins have a choline-binding module (CBM) for anchoring to the choline-containing teichoic acid of the pneumococcal cell wall. This CBM is formed by a repeat of about 20 amino acids and is found in multiple tandem copies (ranging from 4 to 18). The catalytic module of Cpl-1 has an irregular (β/α)6β3 barrel, and the cell wall anchoring module is formed by six similar choline-binding repeats, arranged into two different structural regions: a left-handed superhelical domain configuring two canonical choline-binding sites and a β-sheet domain that bring together the whole structure. This lysozyme is a flexible protein in which the relative position of the catalytic module and the CBM is restrained both by the length of the 10 amino acids extended linker and by hydrophobic interactions between the catalytic barrel and the β-sheet domain. Very recently, small angle X-ray scattering studies [29] showed that, in solution, Cpl-1 keeps the hydrophobic contacts responsible for the intermodular interactions between the catalytic module and the CBM, but modules form a slightly wider angle than in the crystal structure. It has also been pointed out that the plasticity of Cpl-1 structure, aided by the presence of a long and highly hydrophilic linker, might facilitate the simultaneous interaction of the enzyme with the glycanic chain to be hydrolyzed and the choline residues of (lipo)teichoic acids and, so, be relevant for protein function.

Very recently the crystal structure of the catalytic module of PlyB, an endolysin encoded by phage Bcpl, which displays potent lytic activity against the *Bacillus anthracis*-like strain ATCC 4342 has been reported [30]. PlyB comprises an N-terminal catalytic module (PlyBcat) that shares sequence similarity with members of the glycosyl hydrolase family 25 (GH-25) and a cell wall binding module homologous to bacterial SH3b domains. PlyBcat displays an irregular (β/α)β3 barrel (Figure 2), which is similar to that found in other members of the GH-25 family, like Cellosyl (the muramidase from *Streptomyces coelicolor*) [31] and the catalytic module of the Cpl-1 lysozyme [28]. Strict conservation of the catalytic motifs, together with extensive sequence conservation within the deep grooves immediately surrounding the active site suggests that PlyB cleaves the β-1,4-glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine units [30]. The antibacterial activity of PlyB was tested in *vitro*, showing that the full-length PlyB is able to lyse the anthrax strain efficiently. Interestingly, the catalytic module alone, PlyBcat, has a minimal activity, underscoring the importance of the SH3b domain for activity. The specific target of the SH3b domain in PlyB is not known, but it has been shown that the bacterial SH3b domain from the glycol–glycine endopeptidase ALE-1, a homolog of lysostaphin, specifically recognizes inter-peptide penta-glycine bridges [32] within the *S. aureus* PG. However, *Bacillus* spp. does not possess penta-glycine cross-bridges in the PG and therefore an alternative role of the SH3b domain in saccharide binding cannot be excluded.

As mentioned above, relevant information has been provided for another family of endolysins that are not directly dependent on holins for export. In this case, lysozymes are exported by virtue of a SAR domain that functions as a type II signal anchor. This leaves the secreted protein in the periplasm but is tethered to the membrane in an inactive form. When the membrane is depolarized by the holin, the SAR domain exits the bilayer, resulting in activation of the endolysin and cell lysis. Some examples of these proteins include the lysozyme from coliphages Mu, T1, and the endolysins from enterobacteriophage P1 (Lyz) and lambdoid coliphage 21 (R23). All these lysozymes are members of the glycosyl hydrolase family 24 (GH-24). Crystal structures of the inactive and active forms of the Lyz endolysin [22] revealed that membrane release requires intramolecular disulfide...
isomerization and that the alternative disulfide linkage in the two forms of Lyz triggers dramatic structural changes. These results give relevant insights into the mechanism of activation of this family of endolysins. The crystal structure of a very similar endolysin, the lysozyme of phage P22, has also been determined [33]. Although no structural analysis has been reported yet, its three-dimensional structure closely resembles those of Lyz and T4 lysozymes.

The crystal structure and lytic activity of another B. anthracis phage endolysin, PlyL, have been published [34*] (Figure 2). PlyL, the endolysin of the λ prophage Ba02, is also composed of an N-terminal catalytic module and a C-terminal CWBM. The crystal structure of the catalytic module, PlyLcat, reveals that its fold is similar to that of T7 NAM-amidase [35] and other Zn-independent amidas like Citrobacter AmpD [36] but, in addition, this fold also resembles the Drosophila PG

Figure 2

Three-dimensional structures of endolysins. Cartoon representation of the crystal structures of Cpl-1, the catalytic module of PlyB (PlyBcat), Lyz, the lysozyme from phage P22, PlyPSA, and the catalytic module of PlyL (PlyLcat). Catalytic modules are colored in purple, CWBMs in gold and linkers in green. Catalytic Zn cations are depicted as yellow spheres. Scheme comparing the modular composition of endolysins Cpl-1, PlyB, Lyz, lysozyme from phage P22, PlyPSA, and PlyL is shown. Color coding is the same as on the other cartoons.
recognition protein PGRP-LB [37]. The zinc co-ordinating residues are invariant among the Bacillus phage endolysins and therefore it was suggested that all related Bacillus phage endolysins of the PlyL family contain a NAM-amidase domain and thus, share a similar catalytic mechanism. Remarkably, the presence of the CWBM within the full-length PlyL inhibits the lytic activity of the catalytic module [34]. This effect was also observed in a phage endolysin specific to Bacillus cereus (Ply21). It appears that in both cases the CWBM serves as an additional level of selectivity by downregulating, probably by an allosteric mechanism, the catalytic module and allows only the catalytic module to function effectively in the presence of a specific cell wall.

The most recently reported three-dimensional structure of a complete endolysin is from the temperate Listeria monocytogenes phage PSA (PlyPSA) [38]. The two functional modules, providing enzymatic and cell wall binding activities, are connected via a linker segment of six amino acid residues (Figure 2). The N-terminal catalytic module displays a twisted, six-stranded $\beta$-sheet flanked by six helices, similar to that found in the catalytic module of CwIV, a cell wall lytic NAM-amidase from Paenibacillus polymyxa var. colistinus [39]. CwIV is a Zn-dependent peptidase, and the residues involved in cation co-ordination are structurally conserved in PlyPSA. Electron density from a dipeptide ligand was observed in the substrate-binding site; this corroborates that this enzyme is a peptidase even though the biochemical identity of this ligand could not be experimentally confirmed. The CWBM of PlyPSA features a novel fold, comprising two copies of a $\beta$-barrel-like motif, which are held together by means of swapped $\beta$-strand. In contrast to Cpl-1, there are only few interactions between the catalytic and cell wall binding modules in PlyPSA; however, it seems that the rather short linker does not provide much flexibility between the two modules. PlyPSA requires the CWBM to be fully active, indicating that this region is responsible for the efficient binding of the enzyme to its PG substrate. The exact nature of the ligand recognized by the CWBM remains unknown, though the unique structure of the teichoic acid polyribitol-phosphate backbone found in Listeria may be important.

### Structural basis for endolysin cell wall recognition

So far, little is known about the molecular and structural basis for cell wall recognition by endolysins. This knowledge is the key to understand how the bacterial envelope is degraded by endolysins, but it could also shed light on the function and improvement of enzybiotics. The first insight on this structural knowledge has been very recently provided by the X-ray crystal structures of the Cpl-1 lysozyme in complex with three bacterial cell wall PG analogs [40]. In all crystallized Cpl-1 complexes, the PG analogs were located along a groove leading to the Cpl-1 active site (Figure 3). The largest PG analog bound to Cpl-1 (a ($^1$GlcNAc$^2$MurNAc$^3$GlcNAc$^4$MurNAc$^5$)-di-pentapeptide) binds through the three saccharide

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**Figure 3**

Bacterial cell wall recognition by Cpl-1. (a) Superposition of the peptidoglycan structures observed by X-ray crystallography (carbons in gray) and by computation (carbons in black). The protein is drawn as a Connolly solvent-accessible surface. (b) View of a molecule of Cpl-1 depicted as a Connolly surface in green docked onto the cell wall. The peptidoglycan appears in balls (glycan chains) and in sticks (peptide stems).
This model was carried out with group B streptococci [46]. Another important example of this approach is lysostaphin, an endopeptidase produced naturally by Staphylococcus simulans, which efficiently cleaves glycy–glycine bonds in the pentaglycine inter-peptide links of the staphylococcal PG. A single dose of lysostaphin, formulated as a cream, was more effective than a single dose of mupirocin ointment in eradicating S. aureus nasal colonization in a cotton rat model [50]. Furthermore, lysostaphin has been successfully produced in lactobacilli and may have potential for further development.

It is worth emphasizing the extensive studies that have been carried out with Streptococcus pneumoniae, which is currently the leading cause of pneumonia, meningitis, and bloodstream infections in the elderly, infants, and immunocompromised persons and middle ear infections in children. The high prevalence of drug-resistant pneumococci and the limited therapeutic alternatives for successfully treating these resistant organisms make finding new solutions urgent. The variety of in vitro tests and animal models set-up using the different purified preparations of murein hydrolases, either alone, in combination, or together with classical antibiotics, is summarized in Table 1. Pal NAM-amidase and Cpl-1 lysozyme, encoded by the pneumococcal phages Dp-1 and Cp-1, respectively, the only lytic phages isolated so far from pneumococci [25], act very rapidly and specifically against many encapsulated and unencapsulated strains, some of which are highly penicillin-resistant isolates. Remarkably, both enzymes act synergistically when used simultaneously, probably because the combination increases the access to their different cleavage sites of the cell wall [48]. Enzyme cocktails could be especially useful when very rapid lysis is desirable or when working with particular strains that have slower lysis kinetics. Very recently, a new animal model of otitis media has been developed in which infection was established by intranasal injection of a particular piliated pneumococcal strain previously modified to express luciferase [49**]. Once the mucosal surfaces of mice were stably colonized, animals were infected with influenza virus that mimics the disease state. The results demonstrated that Cpl-1 lysozyme could eliminate pneumococcal colonization and prevent the development of otitis media. Additionally, reduction or elimination of resident pathogenic bacteria can prevent secondary bacterial complications of influenza.

The concept of using purified murein hydrolases to kill bacteria by breaking bonds of specific bacterial pathogens has proved to be valid not only with phage-encoded endolysins but also with bacterial enzymes. A successful example of this approach is lysozyme, an endopeptidase produced naturally by Staphylococcus simulans, which efficiently cleaves glycy–glycine bonds in the pentaglycine inter-peptide links of the staphylococcal PG. A single dose of lysozyme, formulated as a cream, was more effective than a single dose of mupirocin ointment in eradicating S. aureus nasal colonization in a cotton rat model [50]. Furthermore, lysozyme has been successfully produced in lactobacilli and may have potential for...
in situ biopreservation in foodstuffs [51]. In a biotechnological application, transgenic cows expressing lysostaphin in their milk were resistant to S. aureus intramammary infection [52]. More recently, mice infected with β-lactam-resistant pneumococcal peritoni- nis-sepsis were effectively cured with intraperitoneal or intravenous injections of LytA NAM-amidase, the major pneumococcal autolysin [53] (Table 1).

**Concerns about endolysin therapy**

Two obvious concerns about the therapeutic use of purified endolysins to kill pathogenic bacteria are related with the resistance and the immune response to these enzymes. Nevertheless, no endolysin-resistant streptococcal bacteria were isolated after repeated exposure to low concentrations of lysins either when grown in liquid media or on agar plates. In this case, the absence of resistance was associated with the presence of specific molecules as cell wall components of the different bacteria: the amino alcohol choline, essential for pneumococcal viability, and polyrhamnose, which has also been shown to be important for streptococcal growth. In pneumococcus, the striking choline-dependence of the murein hydrolases suggested that the presence of this amino alcohol in the cell wall might have exerted strong selective pressure reducing the evolutionary drift of bacterial and phage genes coding for such murein hydrolases [25]. Thus, it is also possible that during the co-evolution between phage and bacteria over the past three to four billion years, the binding domain of their lytic enzymes evolved to target a unique and essential molecule in the cell wall, making resistance to these enzymes a rare event. Targeting phage lysins to an essential bacterial structure gives them an advantage over the small molecule antibiotics to which bacteria can become resistant rather easily.

Enzymes are proteins that can trigger an immune response when delivered both mucosally or systemically, in contrast to antibiotics that are small molecules that are generally not immunogenic. To test an eventual immune response after protein injection, rabbits and mice were hyperimmunized to various endolysins, and the resulting antibodies were tested in western and ELISA assays. The results demonstrated that antibodies did not have the capacity to inactivate the corresponding enzymatic activity, and no signs of anaphylaxis or adverse side effects were observed in the animals [48,54].

**Future prospects**

The knowledge collected for more than 60 years on the molecular biology of phages and on the mechanisms of bacterial pathogenesis will be combined for the most rational development of phages or their products for their use in the treatment and prevention of bacterial infections. On the basis of the most recent studies, there are solid reasons to believe that the development of phage-derived products for treating and preventing bacterial diseases will be successful, at least in limited settings. In this sense, phage therapy received a boost in August 2006, when the US Food and Drug Administration (FDA) approved a cocktail of six individually purified phages that could be used as a food additive and sprayed on meat to kill *Listeria*. Hopefully, another cocktail phage spray

<table>
<thead>
<tr>
<th>Antibacteriala</th>
<th>Study</th>
<th>Animal modelb</th>
<th>Via</th>
<th>Relevant result</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pal</td>
<td>In vitro</td>
<td>Nasopharyngeal colonization</td>
<td>Intranasal</td>
<td>Four logs decrease of viable titer in encapsulated and highly penicillin-resistant strains</td>
<td>[59]</td>
</tr>
<tr>
<td>Cpl-1 + Gen</td>
<td>In vitro</td>
<td>Bacteremia</td>
<td>Intranasal</td>
<td>Synergy against a decreasing penicillin MIC strains</td>
<td>[60]</td>
</tr>
<tr>
<td>Cpl-1 + Pen</td>
<td>In vitro</td>
<td>Otitis media</td>
<td>Intranasal</td>
<td>Complete prevention of otitis media</td>
<td>[49]</td>
</tr>
<tr>
<td>LytA</td>
<td>In vitro</td>
<td>Peritonitis-sepsis</td>
<td>Intraperitoneal</td>
<td>Effective protection from bacteremia and death; synergistic action of both enzymbiotics</td>
<td>[48]</td>
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<tr>
<td>Pal</td>
<td>In vivo</td>
<td>Peritonitis-sepsis</td>
<td>Intraperitoneal</td>
<td>Effective protection from bacteremia and death; synergistic action of both enzymbiotics</td>
<td>[53]</td>
</tr>
<tr>
<td>LytA</td>
<td>In vivo</td>
<td>Peritonitis-sepsis</td>
<td>Intraperitoneal</td>
<td>Four to five logs decrease of bacterial titers in blood and intraperitoneal fluid</td>
<td>[53]</td>
</tr>
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**Table 1**

*Antibacterial studies with pneumococcal murein hydrolases*

*a* Gen, gentamycin; Pen, penicillin; Cfx, cefotaxime.

*b* All experiments used mice except when rats were employed for the endocarditis model.
will be approved by FDA to specifically destroy E. coli O157:H7, a strain that caused several major outbreaks of food-borne illnesses in the past years. These authorizations will pave the way for other phage-based clinical trials, including treatment of human diseases.

As the world now faces a serious challenge in dealing with a host of microbial threats that were once thought to be defeated rather easily by antibiotics, there are many reasons to re-introduce bacteriophage-based therapies as an additional tool in the war against bacterial diseases. These tools will presumably come not only from improved living virions but also from diverse phage-encoded products. In this regard, it is an interesting case of the so-called ‘protein antibiotics’, the gene products of some small phages that do not produce endolysins and are capable of inhibiting the synthesis of the cell wall [55]. Bacteriophages can also be genetically engineered to carry toxic genes/proteins, which can produce cell death without causing lysis, avoiding the unwanted release of bacterial endotoxins [56]. A novel bacteriophage-based strategy has been investigated to reduce the effective dose of antibiotics during treatment holding promise that resensitization of pathogens resistant to a particular antibiotic can be achieved in the presence of phage in vivo [57].

Taking advantage of endolysin specificity for its bacterial target, the genes encoding a specific murein hydrolase able to destroy an intestinal bacterial pathogen can be expressed and secreted in Lactococcus lactis without affecting the probiotic cells [2]. Furthermore, endolysins can be redesigned by protein engineering techniques to improve their killing activity and to modify its target specificity [25,58]. Considering that phage lysins limit the spread of infection and kill targeted bacteria on contact, these enzymes could be used in few years as components of antibacterial sprays, lozenges, mouthwashes, suppositories, inhalers, bandages, and eye drops. Nasal sprays containing the enzymes could be an effective alternative to conventional antibiotics and could help eliminate human reservoirs of the bacteria.

Conclusions

The modern medical community is aware that the grand majority of bacteria living in our bodies are beneficial or innocuous. These bacteria are killed indiscriminately by antibiotics, and their loss exposes tissues to colonization by potentially harmful bacteria. Therefore, the ability of bacteriophages or their components to selectively target pathogenic species of bacteria represents an important advantage compared with antibiotics. The tight binding and potent lytic activity places the endolysins in the category of irreversible inhibitors and promises, at least in principle, a high efficacy with the additional advantage of leaving normal commensal organisms untouched. Since bacteriophages are the most abundant biological entities on earth, they constitute a very rich natural source of potent endolysins. The combination of this enormous natural diversity with the increasing information about their three-dimensional structures and the multiplicity of metagenomic tools envisage a promising scenario to find or to design ‘a la carte’ novel enzybiotics specific to any pathogenic bacterial species.

Acknowledgements

We thank Rubens López, Ernesto García, and Douglas Laurents for critical reading of the manuscript and Inmaculada Pérez-Dorado for help with the preparation of Figure 3. This work was supported by grant SAF2006-00390 and BFU2005-01645 from the Ministerio de Educación y Ciencia. Additional financial support was also provided by the COMBACT program (S-BIO-0260/2006) from the Comunidad Autónoma de Madrid.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

Current Knowledge on Enzybiotics

Hermoso, García and García


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12 Antimicrobials


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