MINIREVIEW

Bacteriophage Endolysins as a Novel Class of Antibacterial Agents

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Endolysins are double-stranded DNA bacteriophage-encoded peptidoglycan hydrolases produced in phage-infected bacterial cells toward the end of the lytic cycle. They reach the peptidoglycan through membrane lesions formed by holins and cleave it, thus, inducing lysis of the bacterial cell and enabling progeny virions to be released. Endolysins are also capable of degrading peptidoglycan when applied externally (as purified recombinant proteins) to the bacterial cell wall, which also results in a rapid lysis of the bacterial cell. The unique ability of endolysins to rapidly cleave peptidoglycan in a generally species-specific manner renders them promising potential antibacterial agents. Originally developed with a view to killing bacteria colonizing mucous membranes (with the first report published in 2001), endolysins also hold promise for the treatment of systemic infections. As potential antibacterials, endolysins possess several important features, for instance, a novel mode of action, a narrow antibacterial spectrum, activity against bacteria regardless of their antibiotic sensitivity, and a low probability of developing resistance. However, there is only one report directly comparing the activity of an endolysin with that of an antibiotic, and no general conclusions can be drawn regarding whether lysins are more effective than traditional antibiotics. The results of the first preclinical studies indicate that the most apparent potential problems associated with endolysin therapy (e.g., their immunogenicity, the release of proinflammatory components during bacteriolysis, or the development of resistance), in fact, may not seriously hinder their use. However, all data regarding the safety and therapeutic effectiveness of endolysins obtained from preclinical studies must be ultimately verified by clinical trials. This review discusses the prophylactic and therapeutic applications of endolysins, especially with respect to their potential use in human medicine. Additionally, we outline current knowledge regarding the structure and natural function of the enzymes in phage biology, including the most recent findings. Exp Biol Med 231:366–377, 2006

Key words: bacteriophage; endolysin; antibiotic-resistance; infection

Introduction

Bacteriophages (bacterial viruses) have developed two basic means of releasing their progeny from bacterial cells. Filamentous phages (e.g., M13, fd, and f1) are continuously extruded from bacterial cells without killing them (1), whereas nonfilamentous bacteriophages induce lysis of the host cell. Lysis is the result of abrupt damage to the bacterial cell wall by means of specific lysis proteins, and can be accomplished in two different ways: (i) inhibition of peptidoglycan synthesis by a single protein (bacteriophages with small single-stranded RNA or DNA genomes; Refs. 2, 3) or (ii) enzymatic cleavage of peptidoglycan by a holin-endolysin system (phages containing large double-stranded DNA [dsDNA] genomes; Refs. 4–6).

Endolysins (also termed lysins) are dsDNA bacteriophage-encoded enzymes produced during the late phase of gene expression in the lytic cycle to degrade peptidoglycan, the main constituent of the bacterial cell wall, thereby enabling progeny virions to be liberated (4, 7). The name endolysin was coined in 1958 to designate a probably

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proteinaceous lytic substance synthesized in bacterial cells during phage multiplication and acting on the cell wall from inside the cell (8). Lysins should, therefore, be clearly distinguished from the lytic enzymes, which, in some phages, are an integral component of the virion and that locally digest the cell wall from the outside to enable the phage genome to be injected into the host cell; an example of such an enzyme is the tail lysozyme of bacteriophage T4 (9). The capability of lysins to digest the cell wall (especially in gram-positive bacteria) when applied exogenously (as recombinant proteins) to bacterial cells has enabled their use as alternative antibacterials. Because of their unique ability to cleave peptidoglycan in a generally species-specific manner, endolysins represent a novel class of antibacterial agents and provide a means of selective and rapid killing of pathogenic bacteria with no effect on the normal microflora. During the past few years, several reports have highlighted the potential of purified recombinant lysins for use in both the prophylaxis and treatment of bacterial infections.

Mode of Action

Because endolysins act on the cell wall, a brief overview of its structure will precede the discussion of their function. The bacterial cell wall protects the cell protoplast from mechanical damage and osmotic rupture (lysis), and is, therefore, essential to bacterial viability. The main constituent of the bacterial cell wall is peptidoglycan (also known as murein; Fig. 1). Peptidoglycan is composed of the repeat polymer of the amino sugars *N*-acetylglucosamine and *N*-acetylmuramic acid, linked together by β-1,4 glycosidic bonds, and tetrapeptide side chains attached to the lactyl group of the muramic acid by amide bonds. Adjacent tetrapeptides may be cross-linked by an interpeptide bond (in gram-negative bacteria) or by an interpeptide bridge (in gram-positive bacteria). In gram-positive bacteria, the cell wall is thick (15–80 nm) and consists of several layers of peptidoglycan associated with teichoic acids. In contrast, the cell wall of gram-negative bacteria is relatively thin (10 nm) and is composed of a single layer of peptidoglycan surrounded by the outer membrane (10).

Depending on the enzymatic specificity, endolysins may be divided into five main classes: (i) *N*-acetylmuramidases (lysozymes), (ii) endo-β-*N*-acetylglucosaminidases, and (iii) lytic transglycosylases, which all cleave the sugar moiety of peptidoglycan; (iv) endopeptidases, which cleave the peptide moiety; and (v) *N*-acetylmuramoyl-L-alanine amidases, which cut the amide bond between both moieties (Fig. 1; Refs. 4, 7, 11). Of these, muramidases and amidases seem to be the most numerous (7). With the exception of transglycosylases (e.g., phage λ lysozyme; Ref. 12), all endolysins are hydrolases (7, 11). As revealed by thin-section electron microscopy, endolysin-mediated peptidoglycan cleavage leads to the formation of holes in the cell wall, through which the high intracellular osmotic pressure extrudes the cytoplasmic membrane, which ultimately results in hypotonic lysis of the bacterial cell (11, 13).

Typically, one endolysin displays only one kind of muralytic activity, i.e., it is a muramidase, a transglycosylase, a glucosaminidase, an endopeptidase, or an amidase. However, at least four bifunctional lysins have also been reported, i.e., enzymes harboring two independent muralytic activities. These endolysins are encoded by *Streptococcus agalactiae* bacteriophage B30 (muramidase and endopeptidase; Ref. 14), *Staphylococcus aureus* phage ϕ11 (endopeptidase and amidase; Ref. 15), *S. agalactiae* phage NCTC 11261 (endopeptidase and muramidase; Ref. 16), and *Staphylococcus warneri* M phage ϕWMY (endopeptidase and amidase; Ref. 17). Another interesting bifunctional lysin is T7 lysozyme, which, notwithstanding its name, is not a muramidase, but rather is an amidase, and which, in addition to its muralytic activity, also binds T7 RNA polymerase and inhibits the transcription of T7 genes during infection of the bacterial cell (18).

Generally, endolysins lack secretory signals, thus, their access to peptidoglycan from inside the cell is dependent on small hydrophobic proteins, termed holins, which enable endolysin molecules to cross the inner membrane. According to the classic paradigm, created mainly on the basis of studies of bacteriophage λ-mediated lysis, both holin and endolysin are produced during the late phase of phage gene expression. Folded endolysin molecules accumulate in the cytosol and, at the genetically determined moment, start to pass through the membrane, likely through homo-oligomeric membrane pores formed by holins. However, it should be stressed that the exact nature of the holin membrane lesion has not yet been defined (4, 19). According to a recent report, holin oligomers may further accumulate in the membrane to form rafts, which would lead to a more generalized membrane disruption than previously thought (20). Furthermore, three endolysins, that is, *Oenococcus oeni* phage fOg44 enzyme Lys44 (21), coliphage P1 lysozyme Lyz (22, 23), and *Lactobacillus plantarum* phage ϕg1e Lys lysin (24), have been found to contain an N-terminal secretory signal and to be translocated across the cytoplasmic membrane by the host *sec* system rather than by holin lesions. In this case, the pre-exported endolysins may be activated by membrane disruption and depolarization mediated by holins (22, 23). Many other lysins are also likely secreted to the periplasm by the host *sec* system, as indicated by N-terminal sequence similarity to Lys44 (21), Lyz (22), and some bacterial autolysins (25). Furthermore, in at least four bacteriophages, that is, the *Bacillus cereus* phages Bastille, TP21, and 12826 (25), and the *Listeria monocytogenes* A511 phage (26), no potential holin-encoding genes are located upstream of the respective endolysin genes; thus, these phage lysins may also be exported to the periplasm by some alternative, nonholin mechanism. In any event, once reaching the peptidoglycan, endolysins rapidly cleave it.

Both endolysin and holin are essential for lysis.

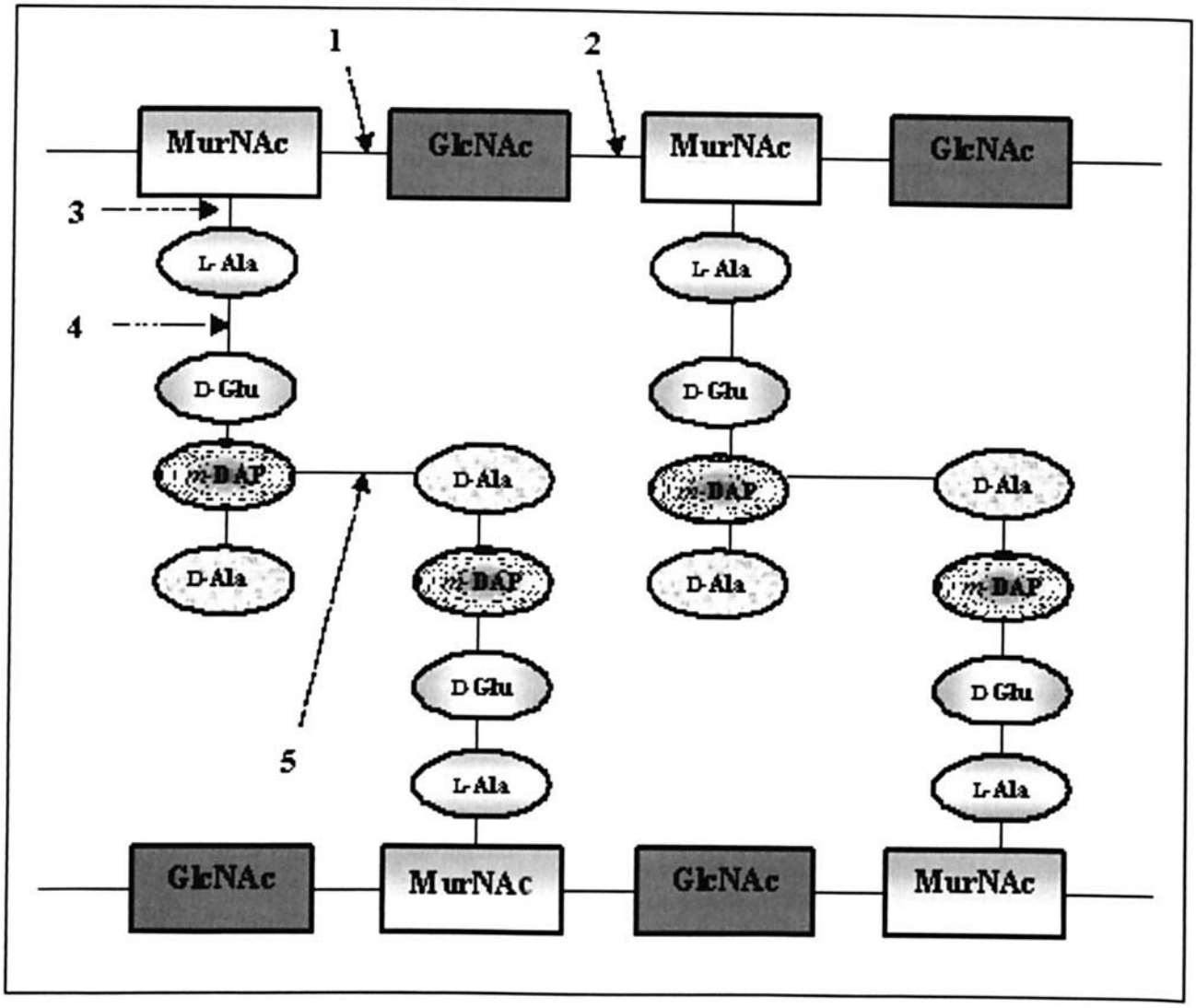

Figure 1. A variant of peptidoglycan typical of many gram-negative bacteria. Peptidoglycan is a heteropolymer of alternating amino sugars *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc), whose adjacent chains are cross-linked by interpeptide bonds between tetrapeptides branching from *N*-acetylmuramic acid. In this case, the tetrapeptides are composed of L-alanine (L-Ala), D-glutamic acid (D-Glu), mesodiaminopimelic acid (m-DAP), and D-alanine (D-Ala), and interpeptide bonds are between m-DAP and D-Ala of adjacent tetrapeptides. The sites of cleavage by the major classes of endolysins are marked with numbers: 1, muramidase (lysozyme) and transglycosylase; 2, glucosaminidase; 3, amidase; 4 and 5, endopeptidase.

Ancillary lysis proteins include Rz and Rz1 (putative endopeptidases attacking the outer membrane or linkages between the membrane and peptidoglycan) and antiholin (a holin inhibitor). Typically, the genes encoding lysis proteins are clustered to form a so-called lysis cassette, in which the holin gene is located immediately upstream of the endolysin gene (4, 19).

In fact, endolysin activity is not essential for killing a bacterial cell by a bacteriophage. After entering bacteria, phages kill their host in the course of multiplication; lysin is a means of destroying the cell wall, thereby enabling progeny virions to be released. Consistent with this, it has been shown that T4 coliphage with the endolysin gene replaced by a green fluorescent protein gene was still capable of killing *Escherichia coli* cells, but could not release its progeny from the bacterial cells (27). Another lysis-deficient virulent phage (LyD, a T4 mutant) also effectively killed bacteria both *in vitro* and *in vivo* (28).

The majority of endolysins, when applied exogenously to bacterial cells (as recombinant proteins), display a narrow spectrum of lytic activity, which may be determined by at least three distinct factors: (i) unique linkages to be cleaved in the cell wall, (ii) specific enzyme activation by components present exclusively in or on the cell wall, and (iii) specificity in substrate recognition and cell wall binding (25). This spectrum is often restricted (with some minor exceptions) to the host bacterial species of the phage from which a certain endolysin was derived (29–31); in some cases, it is genus specific (25, 32). However, amidases have been suggested to display a broader spectrum of antibacterial activity than other classes of endolysins because of the very frequent presence of the amide bond between *N*-

acetylmuramic acid and L-alanine in peptidoglycan (15). Indeed, of the two broader-spectrum lysins reported recently, one, PlyV12 of *Enterococcus faecalis* phage ϕ1, is probably an amidase with an antibacterial spectrum encompassing not only *E. faecalis* and *E. faecium*, but also *Streptococcus pyogenes*, Groups B and C streptococci, and *S. aureus* (33). In contrast, the other enzyme, Mur-LH of *Lactobacillus helveticus* phage ϕ-0303, is not an amidase, but a muramidase, and it exhibits lytic activity against 10 different bacterial species (34). In some cases, the lytic range of an endolysin may considerably exceed that of the phage from which it was derived. Two recent examples include *S. agalactiae* B30 phage lysin (14) and *Clostridium perfringens* phage ϕ3626 Ply3626 amidase (29).

The antibacterial activity of endolysins is commonly ascribed to their enzymatic function, that is, cleavage of the covalent bonds in peptidoglycan (7, 11, 15). However, some lysins, especially those of phages of gram-negative bacteria, are capable of affecting bacterial cells by means of a mechanism completely independent of their enzymatic activity. At least two endolysins, namely T4 lysozyme (35) and the endolysin derived from the *Bacillus amyloliquefaciens* phage (36), have been found to contain sequences in the C-terminus similar to those typical of cationic antimicrobial peptides. In T4 lysozyme, four α-helices have been identified in the C-terminus, of which, three possess amphipathic characteristics, and at least one (α4) exemplifies a typical positively charged amphipathic α-helix, whose basic amino acid residues enable interactions with the negatively charged bacterial outer membrane components. Furthermore, membrane disruption by T4 lysozyme is apparently more important in its bactericidal activity than enzymatic degradation of peptidoglycan, because (i) heat-denaturated T4 lysozyme, lacking enzymatic activity, fully retained its bactericidal activity; (ii) M6K, a mutant T4 lysozyme, showed an approximately 4-fold enhanced bactericidal activity, with no significant increase in enzymatic activity; and (iii) a synthetic peptide with an amino acid sequence corresponding to α4 displayed a strong bactericidal activity with no enzymatic activity (35).

Bacillus amyloliquefaciens phage auxiliary endolysin lys1521 (37) has also been reported to contain two regions in the C-terminus that are rich in positively charged amino acids (arginine and lysine) surrounded by hydrophobic residues. Peptides with sequences corresponding to either region, a fusion peptide composed of both, and a catalytically inactive mutant enzyme have been found to enhance the permeability of the *Pseudomonas aeruginosa* outer membrane. However, in this case, both sequences in an intact enzyme molecule are not capable of inducing *P. aeruginosa* cell lysis by themselves, but rather mediate access of the N-terminal enzymatic domain to peptidoglycan. For full antibacterial activity of this endolysin, both the enzymatic activity and the C-terminal part of the polypeptide chain are essential (36). These findings are of great importance, because they highlight the possibility of also killing gram-negative bacteria by lysins acting on them from outside (see "Resistance" section below).

As opposed to the current, advanced state of research into endolysins bacteriolytic activity, knowledge regarding endolysin receptors on the cell wall remains scanty. The most in-depth study addressing this topic was conducted by Loessner *et al.*, who showed that, in the case of the *L. monocytogenes* phage enzymes Ply118 and Ply500, (i) the endolysin ligands are different from the bacteriophage receptors; (ii) the ligand is a carbohydrate component of the cell wall, probably the entire unique structure of the serovar-specific poly(ribitolphosphate) backbone of teichoic acid; (iii) the binding of the enzymes to their ligands is noncovalent and occurs by means of ionic interaction; (iv) there are 4×10^4 to 8×10^4 endolysin binding sites on a single bacterial cell; and (v) the affinities of the binding domains of the enzymes to their ligands are very high (in the nanomolar range, the affinity constant being 3×10^8 to 6×10^8) and comparable to those of antibacterial affinity-matured antibodies generated during the secondary humoral immune response (32). In the case of endolysins of pneumococcal phages, the receptor is choline, a component of the teichoic acids of the pneumococcal cell wall, which is essential for bacterial viability (38, 39).

Structure

The typical feature of endolysins is their modular structure, that is, they are composed of at least two distinct functional domains (modules), with the catalytic domain(s) typically situated at the N terminus and the cell wall–binding domain at the C terminus (7, 11, 32). This modular structure may be experimentally demonstrated by an analysis of enzymatic and cell wall–binding functions of deletion mutants (15, 32, 40), the creation of functional chimeric enzymes (41), site-directed mutagenesis (14), and x-ray crystallography (39). The data obtained from these (and many other) studies clearly show that the modular structure is a typical feature of the majority of endolysins. However, it should be noted that not all lysins posses a modular structure, an example of such an enzyme being T7 lysozyme, which is a single-domain, globular protein (42).

Interestingly, the C-terminal domain, although responsible for targeting the bacterial cell wall, is not always essential for endolysin antibacterial activity. It has been shown that C-truncated Mur (an endolysin of *Lactobacillus delbrueckii* phage LL-H) retains its lytic activity (43). Furthermore, a C-truncated form of two different staphylococcal phage enzymes (44, 45), *L. monocytogenes* phage A511 Ply511 lysin (46), *Bacillus anthracis* prophage λ Ba02 PlyL enzyme, and *B. cereus* phage TP21 Ply21 endolysin (47), actually exhibited a higher bacteriolytic activity than the full-length enzyme molecule. A possible explanation for these findings is that, in some endolysins, an additional role of the C-terminal domain might be the

inhibition of the catalytic activity of the N-terminal domain when not bound to the cognate cell wall. This inhibitory effect might be relieved on high-affinity binding of the cell wall by the C-terminal domain (47). However, in other lysins, for instance, Ply118 and Ply500 (of *L. monocytogenes* phages A118 and A500, respectively; Ref. 32), *B. amyloliquefaciens* phage endolysin (40), and Ply3626 (a *C. perfringens* phage ϕ3626 lysin; Ref. 29), both domains are required for antibacterial activity.

The modular structure of endolysins seems to be an advantage because it enables one to alter their binding specificity and enzymatic activity independently by replacing either domain with the corresponding domain from another enzyme (41, 48, 49). In some cases, a relatively simple method of improving their catalytic activity could be deletion of a fragment of the C-terminus (44–47). Furthermore, there are several other strategies for engineering enzymes, for instance, random mutagenesis and targeted mutagenesis (50, 51). These methods might also be used for altering basic functions of endolysins.

An interesting question is the evolution of endolysins. It has been postulated, based on studies of *Streptococcus pneumoniae* and its phages, that these enzymes may have evolved by the interchange of phage and bacterial genes encoding individual modules (52). An evolutionary relationship between phage endolysins and bacterial lytic enzymes (autolysins) was originally proposed, based on significant nucleotide sequence similarities (52), and it gained strong support by the creation of functional chimeric phage-bacterial enzymes, that is, enzymes in which one domain was of phage origin and the other domain was of bacterial origin (53, 54), and by the existence of natural chimeric endolysins of intergeneric origin (55). Such a postulated evolutionary relationship between endolysins and autolysins would be another example of horizontal gene transfer between bacteriophages and their host bacterial cells, a phenomenon also reported for other genes (56).

The major task of endolysins is to enable progeny virions to be released from the host cell. In this regard, to ensure the effective liberation of phages, the cell wall–binding domain may have evolved to target some unique and essential component of the cell wall of the host bacteria (11, 13). This would explain the variability of this module and the highly selective lytic activity of endolysins and would imply that resistance should develop rarely (see "Resistance" section below; Refs. 11, 13). However, the similarity of the sequences of the catalytic domains within the major classes of endolysins (11) may be explained, at least in some cases (e.g., of amidases), by the conserved character of the corresponding peptidoglycan bonds (7).

Endolysins as Antibacterials

The feasibility of using of endolysins as antibacterial agents arises from the fact that they display muralytic activity (especially to gram-positive bacteria (see "Resistance" section below), also when added exogenously to bacterial cells. To the best of our knowledge, the capability of a partially purified lysin to kill bacteria was first reported back in 1959 (57). However, it was not until 2001 that it was demonstrated that purified recombinant endolysins may constitute highly effective topical antibacterial agents (58). The most likely reason for this delay was the fact that earlier antibiotic resistance was not a problem serious enough to compel the development of alternative antibacterial agents (59). Several basic applications have been reported for endolysins, including: (i) the elimination of bacterial colonization of mucous membranes, (ii) the treatment of bacterial infections, (iii) the biocontrol of bacteria in food and feed, and (iv) the protection of plants against phytopathogenic bacteria. However, because this review focuses on the antibacterial activity of endolysins with respect to their potential use in human medicine, only the first two applications will be discussed in more detail.

Elimination of Bacterial Colonization of Mucous Membranes. The human mucous membranes are colonized by many potentially pathogenic bacterial species; in the case of the upper respiratory tract, these include *S. pneumoniae*, *Moraxella catarrhalis*, *Haemophilus influenzae*, *Neisseria meningitidis*, and *S. aureus* (60). This colonization is of great importance, because it provides a potential starting point for infection (61, 62). Furthermore, it contributes to the horizontal spread of pathogenic bacteria within the community (63, 64). Considering these facts, it is logical that elimination of mucosal colonization of the upper respiratory tract results in a reduction in the incidence of infections (65) and the community spread of bacteria (66). Prophylactic use of antibiotics in this regard is hindered because of the emergence of resistance and the requirement of multiple drug applications, which precludes rapid elimination of colonization. In view of the prevalence of mucosal colonization (60, 67), the requirement of the development of novel topical antibacterials becomes apparent. During the past few years, several reports have shown that endolysins may be very effective in this regard. In fact, they were originally developed with a view to controlling bacteria colonizing mucous membranes (13, 31, 58).

The first endolysin to be exploited as a topical antibacterial agent was that of Group C streptococci C1 phage (~100 kDa). Interestingly, not Group C, but rather, Group A, streptococci were found to be most sensitive to its lytic activity. The enzyme was found to kill *in vitro* each of the 10 Group A streptococcal strains tested, including the serologic grouping strain, an M-negative strain, 8 distinct M types (representing Class I and Class II streptococci), and an A variant strain. Streptococci of Groups C and E were considerably less susceptible, and streptococci Groups B, D, F, G, L, and N were resistant. Of the eight oral streptococcal species tested, just one, *Streptococcus gordonii*, was slightly sensitive. Three gram-positive species often found in oral microflora and four gram-negative species also proved

completely resistant. Such an antibacterial spectrum seems almost ideal with regard to the use of the enzyme as a decolonizing agent, because it almost selectively kills potentially pathogenic streptococci (Groups A and C) without affecting indigenous oral microflora. The lytic activity of the endolysin was very rapid, as exposure of approximately 10^7 Group A streptococci to 1000 U (10 ng) of the endolysin resulted in complete cell death within 5 secs. Furthermore, just a single dose administered to the oral cavity of mice before 10^7 Group A streptococci administration was sufficient to provide significant protection from colonization of the upper respiratory mucosal epithelium (28.5% infected vs. 70.5% infected in the group that received no treatment). In mice that were colonized despite lysin treatment, colony-forming units (cfu) counts were generally lower, and either the cfus remained low or the colonization was eliminated within 48 hrs. In contrast, all cfus in control mice increased at 48 hrs, and one mouse died. When 500 U of the enzyme was given orally to nine heavily colonized mice (>300 cfu per swab), no streptococci were detected at 2 hrs. However, within 48 hrs, two animals revealed positive cultures and a third animal died, probably because of recolonization by bacteria previously internalized within epithelial cells. Nevertheless, because these bacteria remained sensitive to lysin, it was concluded that periodic treatment with the enzyme might help to eliminate streptococci from the upper respiratory mucosal epithelium (58).

Such encouraging results obtained with a streptococcal endolysin were confirmed for two pneumococcal enzymes. The first was phage Dp-1 Pal amidase (34.6 kDa; Ref. 55). *In vitro*, the enzyme was shown to lyse 15 distinct clinical strains of *S. pneumoniae*, including representatives of the 9 serogroups most frequently causing invasive disease in North America, Europe, Africa, and Oceania, and three strains that are highly resistant to penicillin. The lytic activity of Pal was very rapid; 100 U decreased the viable titer of exponentially growing bacteria by $\log_{10}$ 3.3 to $\log_{10}$ 4.7 cfu/ml within 30 secs (penicillin-resistant strains were killed as effectively as penicillin-sensitive strains). Of the eight species of oral commensal streptococci tested, only *Streptococcus oralis* and *Streptococcus mitis* were sensitive, although to a significantly lesser degree; the other six species were completely resistant. The bacteria were susceptible to lysis regardless of the presence of the capsule, indicating that it is not capable of blocking the enzyme's access to peptidoglycan. Furthermore, because mutants lacking autolysin LytA were equally susceptible to lysis as the clinical strains, this autolysin does not seem to be essential for Pal lytic activity. In a murine model of nasopharyngeal colonization, just one topical dose of Pal completely cleared bacteria from the mucosa (31).

The other pneumococcal enzyme, Cp-1 phage Cpl-1 lysozyme (39 kDa; Ref. 68), also proved to be a highly efficient decolonizing agent. As in the case of Pal, the antibacterial spectrum of Cpl-1 is generally restricted to *S. pneumoniae*; all 19 pneumococcal strains tested, including three highly penicillin-resistant clones, were sensitive. In a murine model of nasopharyngeal colonization, no bacteria were recovered in the nasal wash 5 hrs after one oral or intranasal dose of Cpl-1 (69).

Another enzyme, *S. agalactiae* phage NCTC 11261 PlyGBS (49.6 kDa), was developed as a potential prophylactic against infection by *S. agalactiae* in newborns. *S. agalactiae* is known to colonize the human genitals and lower gastrointestinal tract and can be transmitted to a newborn from its colonized mother before or during delivery. Currently, the first-line agent for intrapartum prophylaxis against *S. agalactiae* neonatal infection is penicillin. However, PlyGBS seems to be a superior decolonizing agent because of its high specificity (especially the lack of activity toward two common vaginal commensals, *Lactobacillus acidophilus* and *Lactobacillus crispatus*), the far lower probability of developing resistance and occurrence of adverse side effects (see "Safety" and "Resistance" sections below), and its very rapid activity. Furthermore, the optimal pH for PlyGBS (~5.0) is within the pH range found in the human vaginal tract. Just a single dose of the enzyme was shown to significantly reduce *S. agalactiae* vaginal and oropharynx colonization in mice. The latter finding is important, because neonatal meningitis is probably initiated through the oropharynx. Thus, PlyGBS may provide not only a means of treating vaginal colonization of pregnant women, but the direct decontamination of newborns as well (16). A similar application was proposed for an endolysin of another *S. agalactiae* phage, B30. *In vitro*, it was active against Groups A, B, C, E, and G streptococci. The fact that the enzyme's optimum pH is similar to that found in pregnant women heavily colonized vaginally with *S. agalactiae* seems to render it suitable for topical intravaginal use. However, its potential as an antibacterial agent has not yet been tested *in vivo* (14).

In the light of the results obtained from these murine models of mucosal colonization, endolysins do seem to be highly promising decolonizing agents. However, as yet, there has been no report directly comparing the therapeutic effectiveness of endolysins and antibiotics in that regard. Nevertheless, it has been shown that a single dose of lysostaphin, an endopeptidase produced by *Staphylococcus simulans* that selectively cleaves the pentaglycine cross-bridge of the *S. aureus* peptidoglycan, formulated in a petroleum-based cream, was more effective than a single dose of mupirocin ointment in eradicating *S. aureus* nasal colonization in a cotton rat model (70). Thus, bacteriophage lytic enzymes are also quite likely to prove superior to antibiotics in this regard. The enzymes are the first antibacterial agents that can rapidly and highly selectively kill pathogenic bacteria regardless of their antibiotic sensitivity, thereby providing a unique means of prophylaxis of bacterial infections.

Treatment of Bacterial Infections. Although the use of endolysins for the treatment of bacterial infections

may theoretically encounter a few very serious problems (see "Immunogenicity" and "Safety" sections below), the first such attempts have already been made. The first reported investigation of such a use of lysin was that by Schuch *et al.*, who used the *B. anthracis* phage γ PlyG amidase (27 kDa). The enzyme was found to lyse *B. anthracis* (all of 14 isolates/strains gathered worldwide) and a single strain (RSVF1) of *B. cereus*, which is closely related to *B. anthracis*. One *B. cereus* strain was only slightly sensitive, whereas 8 other *B. cereus* strains and 12 other species were completely resistant. Capsulated strains of *B. anthracis* also proved susceptible to PlyG's lytic activity. Endospores were degraded only after the induction of germination; in the dormant state, they were resistant. A single 50-U dose of PlyG administered intraperitoneally 15 mins after a lethal dose of RSVF1 cells prevented death in 13 of 19 mice (68.4%). The remaining mice survived for 6–21 hrs, whereas animals in the control group died within 4 hrs. After administration of 150 U of the enzyme, 76.9% of the animals survived. It is anticipated that still higher or multiple doses of the enzyme may yield better results. Results of experiments evaluating the *in vivo* activity of PlyG against *B. anthracis* have not yet been published (11, 30).

Two other endolysins, Cpl-1 and Pal, have been used in the treatment of pneumococcal bacteremia (71). Both were found effective, acting in a dose-dependent manner. The highest single 200-μg dose administered to mice intraperitoneally 1 hr after challenge with a lethal dose of *S. pneumoniae* (a clinical multiresistant isolate) protected 100% of mice, which was paralleled by a sharp drop in bacterial colony counts (by ~4 log units at 2 hrs to practically undetectable levels after 4–5 days); in contrast, the mean bacterial titer in the control group reached approximately 10^7 to 10^8 cfu/ml. The antibacterial effect of both Cpl-1 and Pal was shown to be a protein-specific function, because it was not observed after administration of heat-inactivated enzymes (71).

In another study, a single 2000-μg dose of Cpl-1 administered to mice intravenously 10 hrs after pneumococcal challenge was sufficient to reduce bacterial titers from a median of $\log_{10}$ 4.70 cfu/ml to undetectable levels ($<\log_{10}$ 2.00 cfu/ml) within 15 mins. The same dose of the enzyme administered 1 hr after the challenge rescued 100% of mice from death, as compared with a 20% survival in the control group. In advanced bacteremia, administration of two doses of the enzyme at 5 and 10 hrs still significantly prolonged the survival of mice. However, surviving animals ultimately died, indicating that two doses of the enzyme were not sufficient for complete eradication of the bacteria. The likely reason for this is the short half-life of Cpl-1 (20.5 mins). Nevertheless, even a single dose of the enzyme was sufficient to lower the bacterial titers by ≥99% for at least 2 hrs, which implies that constant intravenous infusion might be enough to eradicate pneumococci completely (69).

A very interesting endolysin has been recently identified by Yoong *et al.*, that is, PlyV12 amidase of the *E. faecalis* phage ϕ1 (~34 kDa). This endolysin is unusual in that it is the first lysin reported to exhibit significant lytic activity against several pathogenic bacterial species, i.e., *E. faecalis* and *E. faecium* (14 clinical and laboratory strains, including 2 vancomycin-resistant *E. faecalis* strains and 3 vancomycin-resistant *E. faecium* strains), *S. pyogenes*, and Groups B and C streptococci. Because of the unusually broad range of sensitive pathogenic bacteria and the number of different infections caused by those bacteria, the endolysin may find several prophylactic/therapeutic applications. However, thus far, its antibacterial activity has only been tested *in vitro* (33).

Two articles were recently published regarding the potential therapeutic use of lysins. The first reports the *in vitro* lytic activity of LysK enzyme against several staphylococcal species, including methicillin-resistant *S. aureus* strains (72). The second concerns the treatment of experimental pneumococcal endocarditis in rats with Cpl-1. In this study, the endolysin was found to kill bacteria more rapidly than vancomycin in both blood and aortic vegetations. However, the administration of Cpl-1 also led to a higher release of various cytokines, including interleukin (IL)-1α and β, IL-6, IL-10, interferon-γ, and tumor necrosis factor-α, compared with antibiotics (73).

It has been shown that the efficacy of lysins may be further enhanced by using a combination of two enzymes of different enzymatic specificities, both *in vitro* (74) and *in vivo* (71). In such a combination, lysins seem to act synergistically on the cell wall (71, 74). Synergy was observed in both penicillin-sensitive and penicillin-resistant bacterial strains (74).

Other Applications. Another potential application of lysins may be the biocontrol of bacteria in food and feed. In this regard, one can use either purified enzymes added directly to food or feed, or recombinant bacteria producing and secreting endolysin molecules. The latter approach was exploited by Gaeng *et al.*, who showed that recombinant *Lactococcus lactis* cells with an introduced listerial endolysin gene could quantitatively secrete functional enzyme molecules, as indicated by the rapid lysis of *L. monocytogenes* cells in the surrounding medium. Secretion of functional lysin was also accomplished in a lactose-using *L. lactis* strain that can be used in the fermentation of milk, which raises the possibility of developing starter lactococcal cultures to selectively protect dairy products against *L. monocytogenes* contamination (46). Another enzyme that may find similar application is the *C. perfringens* phage ϕ3626 Ply3626 amidase (38.8 kDa), which holds promise for use as an anticlostridial agent in food or poultry feed (29).

Some endolysins may also be used to protect plants against phytopathogenic bacteria. To this end, one can create transgenic plants expressing an endolysin gene (75, 76). In this case, the enzyme is secreted to the intercellular spaces of the plant and kills bacteria at a very early stage of

infection (75). Alternatively, recombinant lysin can be applied on the plant surface (77).

Endolysins species-specific bacteriolytic activity also enables one to use endolysins for the rapid detection of bacteria, as reported for *B. anthracis*. In this regard, endolysin-treated germinating *B. anthracis* spores released ATP that could be measured *via* a luminometer as light emitted in the presence of luciferin/luciferase. This method was found to be very sensitive (as few as ~100 germinating spores could be detected), specific, and rapid (2.5×10^3 spores yielded a signal 5 mins after enzyme was added; Ref. 30).

Special Topics Relevant to Endolysin Treatment

Immunogenicity. One of the potential obstacles to endolysin therapy seems to be a humoral immune response induced after both systemic and mucosal administration (78). Such a response could reduce or completely block its antibacterial activity, especially after repeated injections, for instance, during the treatment of a chronic infection. Nevertheless, it has been unexpectedly shown that previous intravenous exposure of mice to Cpl-1 did not significantly diminish its therapeutic efficacy *in vivo*, and hyperimmune rabbit serum only modestly inhibited its activity *in vitro* (69). Also, Jado *et al.* reported that a second dose of this enzyme (administered to mice 10 days after the first dose) may be of equal efficacy (71). Furthermore, antibodies against streptococcal and anthrax phage endolysins obtained from hyperimmunized rabbits did not neutralize their antibacterial activity, likely because of the very high affinity of the enzymes to their substrates in the cell wall (59, 78). This could especially explain the lack of neutralization of the endolysins by antibodies binding the C-terminal domain. However, it remains inexplicable why the antibacterial activity of these enzymes was not blocked by antibodies to the N-terminal catalytic domain (78). In any event, the results of first preclinical studies indicate that the apparent immunogenicity of the endolysins, manifested in the generation of antibodies, in fact, might not preclude their use in the treatment of systemic bacterial infections. In addition, the immunogenicity of lytic enzymes can be considerably reduced by conjugation to polyethylene glycol (PEG), as reported for lysostaphin. PEGylation of a protein is known to reduce antibody binding and uptake by dendritic cells (and hence antigen processing), to prevent the approach of proteolytic enzymes, and to decrease renal ultrafiltration. Thus, predictably, PEGylated lysostaphin, especially that with a low degree of PEG modification, had antibody binding affinity reduced more than 10-fold and a serum half-life of up to 24 hrs, compared with less than 1 hr for the unmodified enzyme. Importantly, a low degree of PEGylation resulted in only a slight decrease in the lytic activity of the enzyme, which can be more than made up for by dramatically improved pharmacokinetics (79). Apparently, similar modification could also be used as a means of reducing the immunogenicity of the endolysins. It is also possible that after administration of the first dose, endolysin could, because of the very rapid lytic activity, kill bacteria before the generation of antibodies.

Safety. With regard to safety, lytic enzymes seem to be innocuous after both topical (31, 58) and systemic (69) treatment in mice. Irritation tests showed that endolysin was nonirritant to the mucosal epithelium after topical administration (58). Both mucosal and skin treatment with lysin specific to Group A streptococci, administered to mice daily for 7 days, did not bring about any histopathologic abnormalities (13). Furthermore, even repeated nasal or intravenous administration of large amounts of the enzyme revealed no signs of toxicity, as assessed by observing the weight, aspect, and behavior of the treated mice for 4 weeks (69). Because peptidoglycan is not found in eukaryotic cells, it is anticipated that lysins will also be well tolerated in humans (11, 13). However, it should be noted that some lytic enzymes, especially endopeptidases, may affect mammalian tissues, as reported for lysostaphin, which has been shown to bind and degrade elastin, because of its high glycine content (80). Although lysostaphin has not been reported to cause any adverse side effects after either systemic (81) or topical (82) administration, this finding still may be important because it raises the possibility that bacteriophage endopeptidases may also be capable of degrading some human proteins.

Another important topic that needs to be addressed with respect to the question of the safety of endolysin treatment is the possibility of the release of various proinflammatory cell wall– and membrane-associated components during bacteriolysis. These include endotoxin, teichoic and lipoteichoic acids, and peptidoglycan, and their massive release might result in serious complications, that is, septic shock and multiple organ failure (83). However, in no endolysin study hitherto conducted have any bacteriolysis-associated side effects been observed (69, 71), nor have they ever been reported to occur during phage therapy of bacterial infections. Although virulent phages used in such treatment also induce lysis of bacterial cells, they do seem to be safe antibacterials (84). Thus, the odds are that bacteriolysis-induced side effects will not restrict the use of endolysins in the treatment of systemic infections.

Resistance. It has been pointed out that the development of resistance to lysins is quite unlikely, because these enzymes, being essential for the release of phage progeny, may have evolved to target unique molecules in the cell wall that are essential for bacterial viability (11, 13, 31). The theory derives support from the fact that the receptor for pneumococcal phage lysins is choline, an amino alcohol found to be necessary for pneumococcal viability (31). Furthermore, polyrhamnose, a molecule that is essential for endolysin binding to Group A streptococci, was also shown to be important for bacterial growth (13). Indeed, to the best of our knowledge, no case of resistance to endolysin has ever been reported. Even repeated exposure of *S. pneumo-*

niae (31) or *B. cereus* (30) to low doses of lysin both on agar plates and in liquid cultures failed to identify resistant mutants. Moreover, it has been found that *B. cereus* subjected to mutagenesis with methanesulfonic acid ethyl ester developed approximately 1,000-fold and 10,000-fold increases in novobiocin and streptomycin resistance, respectively, while remaining sensitive to endolysin (no resistant derivatives were identified; Ref. 30). Thus far, the major factor reported to decrease bacterial susceptibility to endolysin during the stationary phase are likely to be changes in the cell wall structure, such as an increase in peptidoglycan cross-linking, deacetylation of the amino sugars, or an increase in the amount of cell wall–associated proteins and polysaccharides (14, 31). However, this problem is not solely relevant to endolysin treatment, because nonreplicating bacteria are also known to be less susceptible to antibiotics (85) and most lytic phages (86).

Another important question is that concerning the possibility of treating infections caused by gram-negative bacteria. In these bacteria, the peptidoglycan layer is surrounded by the outer membrane, which is impermeable to macromolecules (87) and apparently renders them resistant to endolysin activity (7). However, some endolysins are capable of killing gram-negative bacteria despite the presence of the outer membrane, by means of their C-terminal membrane-active peptide sequences (35, 36). Although such sequences have been found in only two endolysins, it is still possible that they are also present in others. Furthermore, similar sequences may be fused to enzymes that do not possess them by means of genetic engineering, as reported for lysozyme (88).

In addition, endolysins generally seem to be rather thermostable proteins. For example, Cpl-1 was stable for more than 6 months at 4°C, for at least 3 weeks at 37°C, and for at least 30 mins at 45°C (69). Incubation of another enzyme, Mur, at 60°C for 30 mins decreased its activity by only 20% (34). Although the pH optimum for lysins is usually within the range of 4.0–6.0 (14, 16, 33, 43, 69), at least some lysins retain significant antibacterial activity in blood (pH 7.4; Refs. 11, 69, 71).

Endolysins and Antibiotics

We are currently witnessing a very severe antibiotic-therapy crisis, which is the result of three major factors: a dramatic rise in antibiotic resistance among bacteria, a shortage of novel classes of antibiotics, and the withdrawal of the pharmaceutical industry from the discovery and development of new antibiotics (89–92). Thus, there is an urgent need for the development of novel antibacterial agents acting on novel targets in the bacterial cell (90, 92). Endolysins seem to be very promising in this regard, because both their receptors and their modes of action are different from those used by conventional antibiotics. Consistent with this, they have also been shown to be efficient in killing antibiotic-resistant bacteria, including penicillin-resistant *S. pneumoniae* (31, 69, 71), methicillin-resistant *S. aureus* (72), and vancomycin-resistant *E. faecalis* and *E. faecium* (33).

Another significant feature of endolysins, which clearly distinguishes them from antibiotics, is their antibacterial spectrum. In lytic enzymes, the spectrum is narrow, because it generally encompasses the host species of the phage from which the particular enzyme was derived. In contrast, antibiotics have a rather broad spectrum of antibacterial activity. Thus, endolysins are less likely to disturb the balance of indigenous bacterial microflora and cause secondary infections, as is the case with antibiotics (93). Nevertheless, it is known that one of the major factors that regulate the balance of the microflora is interspecies competition, that is, some bacterial species can interfere with the growth of other species (60). Therefore, it is possible that even targeted killing of a single bacterial species may alter the balance by relieving this inhibitory activity.

Importantly, in some cases, endolysins may act synergistically with antibiotics, as reported for Cpl-1 (94). *In vitro*, Cpl-1 showed synergistic activity with penicillin against an extremely penicillin-resistant *S. pneumoniae* strain; in the penicillin-susceptible or intermediate strains, no synergy was observed, although there was a clear shift toward synergistic activity in parallel with an increase in penicillin resistance. Unlike penicillin, gentamicin showed increasing synergy with Cpl-1 with decreasing penicillin minimum inhibitory concentration (MIC), and only with a penicillin-susceptible strain was the synergy perfect. In contrast, two other antibiotics tested (levofloxacin and azithromycin) did not act synergistically with Cpl-1. It also remains to be shown whether the synergistic activity of Cpl-1 and some antibiotics found *in vitro* will occur *in vivo* (94).

Concluding Remarks

Quite unexpectedly, endolysins, the phage enzymes known for longer than 40 years, were used in 2001 as topical antibacterial agents and proved to be highly effective in this regard. Since then, several reports have evaluated their potential for both topical and systemic use. From the investigations conducted to date, a clear picture of endolysins is emerging, showing them to be a novel class of antibacterial agents possessing several typical features, the most important being: (i) very rapid and potent antibacterial activity both *in vitro* and *in vivo*, especially against gram-positive bacteria; (ii) a completely new mode of action, that is, enzymatic cleavage of peptidoglycan (hence the proposed name "enzybiotics"; Ref. 58); (iii) activity against bacteria regardless of their antibiotic sensitivity; (iv) a narrow antibacterial spectrum; (v) low probability of developing resistance; (vi) apparent safety; and (vii) relatively easy modifications by means of genetic engineering. These features certainly render endolysins very promising potential antibacterial agents and warrant their

development. Theoretically, their immunogenicity and the possibility of the release of different proinflammatory components during bacteriolysis might restrict their use in the treatment of systemic bacterial infections. However, a considerable body of evidence also indicates that such use is not precluded. Thus, at the current preclinical stage of development, endolysins seem to hold promise both as topical and as systemic antibacterial agents.

It should also be pointed out that bacteriophages are the most abundant group of biologic entities on Earth, generally outnumbering bacteria by several to tens of orders of magnitude (95, 96). Thus, their lysis proteins constitute a very rich natural source of potent antibacterial agents, enabling one likely to find enzymes specific to any pathogenic bacterial species. Especially in view of the urgent need for novel antibacterials, this source certainly should be exploited.

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