CHAPTER 1

ENZYBIOTICS AND THEIR POTENTIAL APPLICATIONS IN MEDICINE

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1. INTRODUCTION

Over the last decade, a dramatic increase in the prevalence of antibiotic resistance has been noted in several medically significant bacterial species, especially *Pseudomonas aeruginosa*, *Acinetobacter baumanii*, *Klebsiella pneumoniae*, as well as *Staphylococcus aureus*, coagulase-negative staphylococci, enterococci, and *Streptococcus pneumoniae* (Hawkey 2008). This unfavorable situation is further aggravated by a shortage of new classes of antibiotics with novel modes of action that are essential to contain the spread of antibiotic-resistant pathogens (Livermore 2004). In fact, some infectious disease experts have expressed concerns that we are returning to the pre-antibiotic era (Larson 2007). Therefore, there is an urgent need to develop novel antibacterial agents to eliminate multidrug-resistant bacteria (Breithaupt 1999). A very interesting class of novel (at least in terms of their formal clinical use) antibacterials are enzybiotics.

The term "enzybiotic" was used for the first time in a paper by Nelson et al. (2001) to designate bacteriophage enzymes endowed with bacterial cell wall-degrading capacity that could be used as antibacterial agents. While some authors suggest that this name should refer to all enzymes exhibiting antibacterial and even antifungal activity (Veiga-Crespo et al. 2007), in this chapter we will discuss only bacterial cell wall-degrading

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enzymes (regardless of their source). Other names that are used with respect to enzybiotics are lytic enzymes and peptidoglycan hydrolases. The latter refers to the major mode of action of enzybiotics, that is, the enzymatic cleavage of peptidoglycan covalent bonds, which results in the hypotonic lysis of a bacterial cell. Peptidoglycan hydrolases constitute an abundant class of enzymes and may be obtained from different sources, for instance, bacteriophages (lysins) and bacteria themselves (bacteriocins and autolysins). Yet another example of well-known enzybiotics are lysozymes, including hen egg white lysozyme and human lysozyme (a list of representative enzybiotics is shown in Table 1.1).

In view of the ever-increasing antibiotic resistance of bacteria, the most important characteristics of enzybiotics are a novel mode of antibacterial action, different from those typical of antibiotics, and the capacity to kill antibiotic-resistant bacteria (Borysowski et al. 2006). Another significant feature of some lytic enzymes is the low probability of developing bacterial resistance (in some cases, the development of enzybiotic resistance results in a reduction in bacterial fitness and virulence; Kusuma et al. 2007).

The goal of this chapter is to discuss the major groups of enzybiotics, including lysins, bacteriocins, autolysins, and lysozymes, in the context of their potential medical applications.

2. LYSINS

2.1. General Features

Lysins or endolysins are double-stranded DNA bacteriophage-encoded enzymes that cleave covalent bonds in peptidoglycan (Borysowski et al. 2006; Fischetti 2008). They are naturally produced in phage-infected bacterial cells during the course of lytic cycle. At the last stage of the cycle, endolysin molecules degrade peptidoglycan, thereby causing lysis of the bacterial cell and ensuring the release of progeny virions (Young et al. 2000). The term "endolysin" was introduced to the scientific literature by F. Jacob and C. R. Fuerst to stress that enzyme molecules act on peptidoglycan from within the bacterial cell in which they are synthesized (Jacob and Fuerst 1958). In view of this, it appears that recombinant enzymes acting on the cell wall from outside the cell (e.g., those used for therapeutic purposes) should be referred to as lysins rather than endolysins. Still another name proposed to designate a lysin is "virolysin," which is intended to point out the viral origin of these enzymes (Parisien et al. 2008). However, this name has not gained popularity and is used very rarely.

TABLE 1.1. A List of Representative Enzybiotics

Enzybiotic Name	Enzybiotic Class	Source	Enzymatic Specificity	Antibacterial Range	Reference
PlyC	Lysin	Phage C1	Amidase	*S. pyogenes*, groups C and E streptococci	Nelson et al. 2006
Pal	Lysin	Phage Dp-1	Amidase	*S. pneumoniae*	Loeffler et al. 2001
Cpl-1	Lysin	Phage Cp-1	Muramidase	*S. pneumoniae*	Loeffler et al. 2003
PlyGBS	Lysin	Phage NCTC 11261	Endopeptidase muramidase	*S. agalactiae*, groups A, C, G, L streptococci	Cheng et al. 2005
Phage B30 lysin	Lysin	Phage B30	Endopeptidase muramidase	*S. agalactiae*, groups A, B, C, E, G streptococci, *E. faecalis*	Baker et al. 2006
LambdaSa1 prophage lysine	Lysin	Prophage LambdaSa1	Endopeptidase	?	Pritchard et al. 2007
LambdaSa2 prophage lysine	Lysin	Prophage LambdaSa2	Endopeptidase glucosaminidase	*S. pyogenes, S. dysgalactiae*, group E streptococci, *S. equi*, group G streptococci, *S. agalactiae*	Pritchard et al. 2007
PlyG	Lysin	Phage γ	Amidase	*B. anthracis*	Schuch et al. 2002
PlyL	Lysin	Prophage λBa02	Amidase	*B. cereus* *B. anthracis*	Low et al. 2005
PlyPH	?	?	?	*B. anthracis*	Yoong et al. 2006
PlyB	Lysin	Phage BcpI	Muramidase	*B. anthracis*	Porter et al. 2007
Ply118	Lysin	Phage A118	Peptidase	*Listeria*	Loessner et al. 2002
Ply500	Lysin	Phage A500	Peptidase	*Listeria*	Loessner et al. 2002

TABLE 1.1. ***Continued***

Enzybiotic Name	Enzybiotic Class	Source	Enzymatic Specificity	Antibacterial Range	Reference
Ply3626	Lysin	Phage Ø3626	Amidase	*C. perfringens*	Zimmer et al. 2002
PlyV12	Lysin	Phage Φ1	Amidase	*E. faecalis, E. faecium, S. pyogenes*, group B, C, E, G streptococci	Yoong et al. 2004
Lyt A	Autolysin	*S. pneumoniae*	Amidase	*S. pneumoniae*	Rodriguez-Cerrato et al. 2007
lysostaphin	Bacteriocin	*S. simulans*	Endopeptidase	*S. aureus*, coagulase-negative staphylococci	Patron et al. 1999
zoocin A	Bacteriocin	*S. equi*	Endopeptidase	*S. equi, S. pyogenes, S. mutans, S. gordonii*	Simmonds et al. 1995
hen egg white lysozyme	Lysozyme	Hen's egg white	Muramidase	Gram-positive bacteria	Sava 1996
		Ø3626			et al. 2002

The table does not include staphylococcal phage lysins that are discussed in Chapter 7.

The main mode of antibacterial action of lysins is the enzymatic cleavage of the covalent bonds in peptidoglycan. Depending on their enzymatic specificities, lysins fall into five major classes: *N*-acetylmuramoyl-L-alanine amidases, endopeptidases, *N*-acetylmuramidases (lysozymes), endo-β-*N*-acetylglucosaminidases, and lytic transglycosylases (Fig. 1.1).

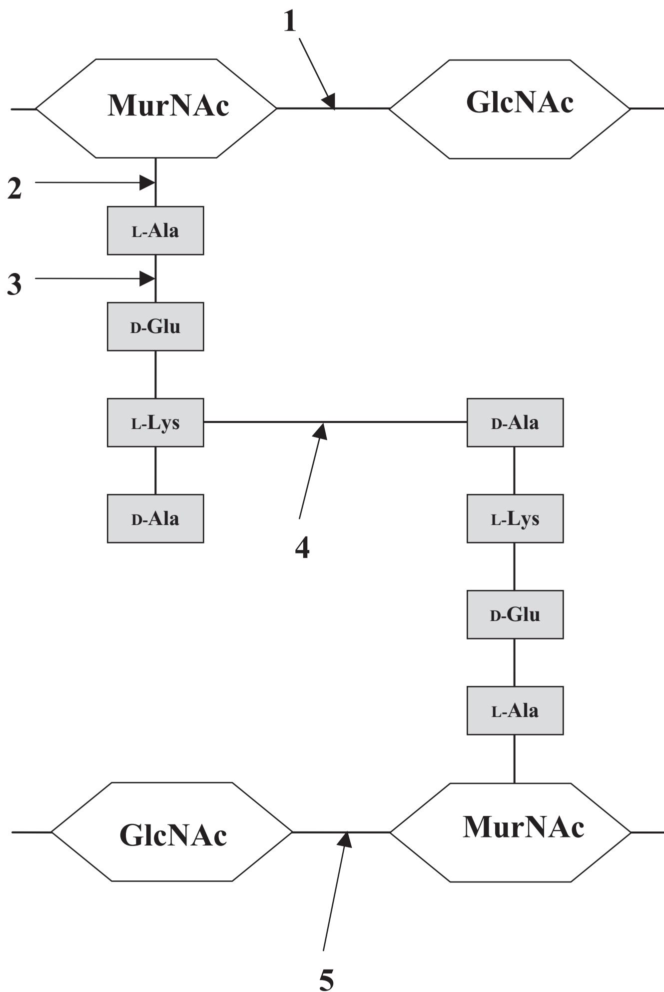

Figure 1.1. Sites of peptidoglycan cleavage by main classes of enzybiotics. This variant of peptidoglycan is typical of *S. aureus*. The backbone of peptidoglycan consists of alternating residues of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc). The tetrapeptide side chains branching off from *N*-acetylmuramic acid are cross-linked by the pentaglycine bridges. The sites of cleavage by enzybiotics with different enzymatic specificities are indicated by the numbered arrows: (1) muramidases and transglycosylases; (2) amidases; (3 and 4) endopeptidases; (5) glucosaminidases.

The majority of lysins described to date exhibit only one kind of muralytic activity, whereas relatively few possess two separate enzymatic domains (Borysowski et al. 2006).

While the main mode of antibacterial activity of lysins is based on enzymatic cleavage of peptidoglycan, it is noteworthy that some of them can also affect bacterial cells by a nonenzymatic mechanism. This mechanism relies on destabilization of the bacterial cytoplasmic membrane by amino acid sequences whose properties, especially their amphipathic secondary structure, positive charge, and hydrophobicity, are similar to those found in cationic antimicrobial peptides (CAPs). Such sequences were identified in T4 phage lysozyme and lysins encoded by *Pseudomonas aeruginosa* phages D3 and ΦKZ (Düring et al. 1999; Rotem et al. 2006). In a series of elegant experiments, these sequences were shown to be more important for T4's antibacterial activity than the enzymatic cleavage of peptidoglycan (Düring et al. 1999). As mentioned above, sequences having physicochemical characteristics typical of CAPs (X1 and Z1) are also contained within lysins encoded by two *P. aeruginosa* phages (Rotem et al. 2006). It was shown that synthetic peptides with amino acid sequences corresponding to X1 and Z1, as well as their shorter analogs, inhibited the growth of several Gram-positive bacterial species in a mechanism analogous to that of CAPs. The authors of the study suggested that endolysins of other phages could also be a source of novel antimicrobial peptides. Another unusual enzyme is the *Bacillus amyloliquefaciens* bacteriophage auxiliary lysin lys1521, whose positively charged C-terminal sequences were shown to increase the permeability of the *P. aeruginosa* outer membrane, thereby facilitating the access of the N-terminal enzymatic domain to peptidoglycan (Muyombwe et al. 1999; Orito et al. 2004).

A typical feature of lysins is their modular structure, which means that they are composed of at least two distinct domains: an N-terminal catalytic domain and a C-terminal bacterial cell wall-binding domain (Loessner et al. 2002; Loessner 2005). As mentioned above, some lysins possess two different catalytic domains. In some lysins both the catalytic and the cell wall-binding domain are indispensable for their lytic activity, while others can lyse bacteria also in their C-truncated forms, although it is the C-terminal domain that is responsible for binding to the bacterial cell wall. Interestingly, lysins were also reported to exhibit higher antibacterial activity after removing their C-terminal domains. These findings are very important because they indicate that the antibacterial activity of some lysins could be increased by simply removing their cell wall-binding domains (Borysowski et al. 2006).

Another typical feature of the vast majority of lysins described to date is a narrow antibacterial range when acting on the bacterial cell from outside. This range is usually limited to the host bacterial species of the bacteriophage encoding the given enzyme. However, it needs to be stressed that lysins are most often capable of killing the majority of strains within a given bacterial species (Fischetti 2008). For instance, Pal, an amidase encoded by *S. pneumoniae* phage Dp-1, was shown to lyse 15 out of 15 pneumococcal strains tested (Loeffler et al. 2001). Another lysin, Ply3626 of *Clostridium perfringens* bacteriophage Ø3626, could also kill all of the 48 *C. perfringens* strains tested (Zimmer et al. 2002). This feature clearly sets lysins apart from lytic phages, which are usually capable of infecting and killing only a small subset of strains within a given bacterial species. Very few lysins were reported to possess a broader antibacterial spectrum (Yoong et al. 2004).

A unique medical application of lysins may be the specific elimination of pathogenic bacterial species (e.g., *S. aureus*) colonizing mucous membranes without adversely affecting normal microflora. Such bacteria can, in some clinical settings, be a starting point for infections (Bogaert et al. 2004; Wertheim et al. 2005). Lysins could thus provide a basis for a novel strategy for preventing some bacterial infections. Furthermore, elimination of the mucosal reservoir of bacteria could contribute to containing the horizontal spread of bacterial pathogens in some communities (Fischetti 2003). Lysins appear to be better decolonizing agents than antibiotics owing to their species-specific and rapid antibacterial activity, capacity for killing antibiotic-resistant bacteria, and lower risk of developing resistance (Fischetti 2003; Cheng et al. 2005). Moreover, a considerable body of experimental data shows that lysins, in spite of their apparent immunogenicity, may also be successfully used in the treatment of systemic bacterial infections and are in this regard effective even after repeated administration (Loeffler et al. 2003; Borysowski et al. 2006).

Discussed below are lysins specific to medically significant bacterial species, including *Streptococcus pyogenes* (group A streptococci), *S. pneumoniae*, *Streptococcus agalactiae* (group B streptococci), *S. aureus*, and *Bacillus anthracis*. However, it needs to be stressed that specific lysins can be most likely obtained for any Gram-positive bacterial pathogen from dsDNA bacteriophage (Schuch et al. 2002). Gram-negative bacteria are essentially resistant to recombinant lytic enzymes due to the presence of the outer membrane (see subsection 6.2). Of particular importance is that lysins are also capable of killing antibiotic-resistant bacteria, as shown for penicillin-resistant *S. pneumoniae*

(Loeffler et al. 2001), vancomycin-resistant *Enterococcus faecalis* and *Enterococcus faecium* (Yoong et al. 2004), as well as methicillin-resistant *S. aureus* (MRSA; O'Flaherty et al. 2005) and *S. aureus* strains with reduced susceptibility to vancomycin (Rashel et al. 2007). It was also shown that lysins can act synergistically with other lytic enzymes and antibiotics (Loeffler and Fischetti 2003; Djurkovic et al. 2005; Becker et al. 2008).

***2.1.1. Lysins Specific to* S. pyogenes** The first and hitherto only lysin specific to *S. pyogenes* that was evaluated as a potential antibacterial agent was PlyC amidase derived from group C streptococci C1 phage (Nelson et al. 2001; Nelson et al. 2006). This enzyme is very interesting in at least two respects. First, it is the most potent lysin reported so far, its activity being over two orders of magnitude higher than those of other bacteriophage lytic enzymes. Second, PlyC is the only known multimeric lysin, while all the others are synthesized as single polypeptides. Although PlyC was first reported in 1957, it was not until 2001 that its antibacterial activity was studied in more detail both *in vitro* and *in vivo*. In fact, it is the first lysin whose activity was studied with a view to potential prophylactic or therapeutic use. *In vitro* experiments revealed that, unlike C1 phage, the enzyme lyses *S. pyogenes* most efficiently, while its activity against groups C and E is substantially lower. All 10 *S. pyogenes* strains tested were efficiently lysed by PlyC. On the other hand, the lysin practically did not act on streptococci groups B, D, F, G, L, and N or other bacterial species with the exception of *Streptococcus gordonii*, which was lysed very slowly. Such an antibacterial range appears to be very advantageous because it is essentially limited to pathogenic streptococci (groups A and C). In a murine model of oral colonization, a single dose of the lysin administered to the oral cavity of mice prior to 10^7 colony forming units (cfu) of group A streptococci resulted in significant protection from the mucosal colonization (only 28.5% of the mice that received PlyC were colonized compared with 70.5% of the animals in the control group). Importantly, in most mice that were colonized despite administration of enzyme, cfu counts remained low throughout the experiment or the bacteria were completely eliminated within 48 h, whereas those in the control group increased during the same period of time. In another experiment, no streptococci were detected in oral swabs of nine heavily colonized mice 2 h after administration of one lysin dose. However, in some animals recolonization was noted within 48 h, which was caused most likely by bacteria previously internalized in epithelial cells of the mucous membrane. Importantly, isolated bacteria were sensitive to

PlyC, which practically rules out resistance to the enzyme's lytic activity as the reason for recolonization.

***2.1.2. Lysins Specific to* S. pneumoniae** Two lysins are currently being developed as potential anti-pneumococcal agents. The first is Pal amidase, encoded by the pneumococcal bacteriophage Dp-1 (Loeffler et al. 2001). *In vitro* experiments showed that Pal is capable of lysing all of the 15 clinical strains of *S. pneumoniae* tested, each of which represented a distinct serotype. Penicillin-resistant strains were lysed as efficiently as penicillin-sensitive ones. Moreover, it was found that the capsule could not block Pal's access to peptidoglycan. Apart from *S. pneumoniae*, only *Streptococcus oralis* and *Streptococcus mitis* were slightly susceptible to the enzyme's lytic activity, while six other streptococcal species belonging to the oral microflora were resistant. In a murine model of nasopharyngeal colonization, one topical dose of lysin administered to mice 42 h after pneumococci was sufficient to completely clear the bacteria from the surface of the mucous membrane. While the administration of a lower dose of Pal did not result in complete elimination of bacteria in all mice, the titers of surviving pneumococci were too low to successfully recolonize the mucous membrane.

The other anti-pneumococcal enzyme is Cpl-1 muramidase of Cp-1 phage (Loeffler et al. 2003). As was the case with Pal, the antibacterial activity of Cpl-1 is essentially specific to *S. pneumoniae*. In a murine model of nasopharyngeal colonization, a single topical dose of enzyme completely eliminated pneumococci from the mucous membrane. The high effectiveness of Cpl-1 as a topical decolonizing agent was confirmed in very interesting experiments performed on a novel murine model mimicking the natural development of secondary acute otitis media (AOM) following viral infection in children (McCullers et al. 2007). In this unique model, a pneumococcal strain engineered to express luciferase was used, which allowed monitoring infection with the use of bioluminescent imaging, was used. To evaluate the efficacy of Cpl-1, mice were colonized intranasally with bacteria and subsequently infected with influenza virus to trigger a secondary pneumococcal AOM. Administration of two topical doses of Cpl-1 resulted in complete elimination of the bacteria in 90% of the mice, while enzyme buffer administered to the mice in the control group had no effect on intranasal pneumococci. Furthermore, no mouse treated with Cpl-1 developed a secondary AOM following viral inoculation, compared with 80% of mice from the control group. It was also shown that AOM can be prevented not only by a complete elimination of colonization,

but also by its partial reduction. The results of this study indicate that anti-pneumococcal lysins could provide a novel means of prophylaxis of secondary AOM in children.

The antibacterial effects of Pal and Cpl-1 were also evaluated in experimental models of different pneumococcal infections, including bacteremia, endocarditis, and meningitidis. In a murine model of pneumococcal bacteremia, a 200-μg dose of either enzymes administered to mice 1 h after inoculation with a lethal dose of multiresistant *S. pneumoniae* rescued 100% of the mice. Cpl-1 and Pal exerted a synergic effect in terms of improving survival rates of the infected mice and synergy was found with different doses and administration times of the enzymes. The antibacterial activity of the enzymes in blood was very rapid, as indicated by a sharp decrease in bacterial titers: ~4 log units 2 h after administration of 200 μg of enzyme. On the 4th and 5th day post-administration, bacteria were either undetectable or their titers in blood were very low, while the mean bacterial titer in the blood of control mice was ~10^7–10^8 cfu/mL. It was also shown that only functional lysin was capable of curing infection, while heat-inactivated enzyme did not have any positive effect. This indicates that the antibacterial effects of the studied enzymes were based on direct killing of bacteria rather than an induction of antibacterial immune response (Jado et al. 2003). The high efficacy of Cpl-1 was confirmed in another study performed on a murine model of bacteremia due to a penicillin-sensitive strain of *S. pneumoniae* (Loeffler et al. 2003).

The antibacterial effects of Cpl-1 were also evaluated in a rat model of endocarditis induced by penicillin-resistant *S. pneumoniae* (Entenza et al. 2005). In this study, two dosing regimens of Cpl-1 were compared. In the first, 16 h after pneumococcal challenge, rats received an intravenous (i.v.) bolus of 10 mg/kg of the enzyme followed by continuous infusion of 5 mg/kg/h for 6 h. This regimen resulted in only a temporary decrease in bacterial titers in blood and failed to reduce vegetation titers. In the other regimen, rats were administered an i.v. bolus of 250 mg/kg followed by continuous infusion of 250 mg/kg/h for 6 h. In this case, the bacteria were cleared from the blood within 30 min and an almost complete eradication was maintained for 6 h. Moreover, a significant decrease in vegetation bacterial titers was noted 30 min after administration of lysin. It was also found that the antibacterial effects of Cpl-1 were much more rapid than those of vancomycin with respect to decreasing bacterial titers in both blood and vegetations (differences between groups that received Cpl-1 and vancomycin were statistically significant at 6 h after administration of either drug). On the other hand, the levels of different pro-inflammatory cytokines in blood were

lower in the rats that received vancomycin than in those that were administered Cpl-1.

Cpl-1 was also used in a model of experimental pneumococcal meningitis in infant rats (Grandgirard et al. 2008). Rats were inoculated with pneumococci intracisternally (i.c.) and the enzyme was administered either i.c. or intraperitoneally (i.p.). Following administration of one i.c. dose of Cpl-1 (~20 mg/kg), bacterial titers in the cerebrospinal fluid (CSF) dropped by three orders of magnitude within 30 min. Although pneumococci were essentially undetectable in the CSF for the next 2 h, their titers started to grow shortly thereafter. The half-life of Cpl-1 in the CSF was about 16 min, and the enzyme was present in the CSF for 2 h after administration of the single dose. It is very likely that the efficacy of Cpl-1 could be higher if its bioavailability in the CSF were increased (e.g., by administering repetitive doses). However, the authors failed to verify this experimentally because repetitive injections were too harmful to the rats. In another experiment, infected rats were administered one i.p. dose of Cpl-1 (200 mg/kg), which resulted in a reduction of pneumococcal titers in the CSF by 98% within 2 h. For 3 h after administration, the concentration of Cpl-1 in the CSF was within the range of 7–12 μg/mL.

***2.1.3. Lysins Specific to* S. agalactiae** Thus far, four lysins derived from *S. agalactiae*-specific phages have been reported. These are bacteriophage NCTC 11261 PlyGBS lysin, B30 phage lysin, and enzymes encoded by LambdaSa1 and LambdaSa2 prophages. While the enzymatic specificity of PlyGBS was not shown directly, it contains two putative catalytic domains: endopeptidase and muramidase. An interesting feature of this lysin is its relatively broad antibacterial range, encompassing, aside from *S. agalactiae*, groups A, C, G, and L streptococci (Cheng et al. 2005).

The second enzyme, phage B30 lysin, was shown to possess two separate enzymatic domains: an N-terminal cysteine, histidine-dependent aminohydrolases/peptidases (CHAP) domain (endopeptidase) and an Acm domain (muramidase) situated in the central part of the protein. Like PlyGBS, bacteriophage B30 lysin can lyse, aside from *S. agalactiae*, other bacteria, including groups A, B, C, E, and G streptococci, as well as *E. faecalis* (Pritchard et al. 2004; Baker et al. 2006).

The last two lysins, that is, those encoded by LambdaSa1 and LambdaSa2 prophages, display γ-D-glutaminyl-L-lysine endopeptidase activity, and the latter also β-D-*N*-acetylglucosaminidase activity (Pritchard et al. 2007; Donovan and Foster-Frey 2008). LambdaSa2 prophage lysin is unusual in that its two-tandem Cpl-7 cell wall-binding

domains are situated not at the C-terminus of the polypeptide chain, but rather between the two enzymatic domains. In turbidity reduction assays, the enzyme was found to act potently on several streptococcal species, including *S. pyogenes*, *Streptococcus dysgalactiae*, group E streptococci, *Streptococcus equi*, and group G streptococci, while its lytic activity against *S. agalactiae* was moderate, in spite of the fact that it is encoded by an *S. agalactiae* prophage. Interestingly, a truncated form of the lysin containing the endopeptidase domain and two Cpl-7 domains had higher lytic activity than the full-length enzyme, but only against some bacteria (*S. agalactiae*, *S. dysgalactiae*, *Streptococcus uberis*, and *S. aureus*). A truncated construct containing the endopeptidase domain and one Cpl-7 domain was less active, whereas the endopeptidase domain lacking any cell wall-binding domain was virtually inactive as were all constructs containing only the glucosaminidase domain regardless of the presence or absence of the Cpl-7 domains. An interesting feature of this enzyme is that it maintains substantial lytic activity across a broad range of pH values (5.5–9.5) (Donovan and Foster-Frey 2008).

The major potential application proposed for these enzymes is intrapartum prophylaxis of early onset neonatal infections caused by *S. agalactiae* colonizing the genital tract (Pritchard et al. 2004; Cheng et al. 2005). It appears that lytic enzymes might be in several respects superior to penicillin, which is currently the first-line agent employed in intrapartum antibiotic prophylaxis. Their first advantage is a relatively higher specificity to *S. agalactiae*, especially their lack of activity against species belonging to the vaginal microflora, such as *Lactobacillus acidophilus* and *Lactobacillus crispatus*. Other characteristics of lysins favoring them over antibiotics include their rapid antibacterial activity as well as low probability of developing resistance and causing side effects. Importantly, the optimal pH values for at least some of them fall within the range typical of the human vaginal tract. While the pH optimum of B30 phage lysin (5.5–6.0) is less than the value of the normal vaginal pH (4.5), it does fall within the pH range likely to occur in women heavily colonized with *S. agalactiae* (Pritchard et al. 2004).

The only *S. agalactiae*-specific lysin whose efficacy was evaluated *in vivo* is PlyGBS (Cheng et al. 2005). In a murine model of vaginal colonization, administration of one topical dose of lysin resulted in approximately 3-log decrease in the bacterial level compared with mice in the control group. One topical dose of PlyGBS was also sufficient to significantly reduce bacterial colonization of the oropharynx mucosa. These results are very important in view of the fact that neonatal *S. agalactiae* meningitidis is likely initiated through the oropharynx. Thus it appears that *S. agalactiae*-specific lytic enzymes might be used not

only to eliminate vaginal colonization in pregnant women before delivery, but also to decontaminate newborns, thereby decreasing the incidence of neonatal infections. These enzymes could be administered topically in a recombinant form or secreted in the genital tract by engineered bacteria.

***2.1.4. Lysins Specific to* S. aureus** Thus far, several lysins encoded by *S. aureus* phages have been described, including MV-L, LysK, PlyTW, Ply187, and *S. aureus* Ø11 phage lysin (Loessner et al. 1998; Loessner et al. 1999; Navarre et al. 1999; O'Flaherty et al. 2005; Rashel et al. 2007). At least some of them can also lyse, aside from *S. aureus*, coagulase-negative staphylococci (O'Flaherty et al. 2005). Of particular importance is that they are also capable of killing MRSA and *S. aureus* strains with reduced susceptibility to vancomycin (Rashel et al. 2007). Some of them have been successfully used in experimental models of staphylococcal infections. These are discussed in detail in Chapter 7.

***2.1.5. Lysins Specific to* B. anthracis** The first *B. anthracis*-specific lysin tested as a potential antibacterial agent was PlyG (Schuch et al. 2002). This enzyme, a putative amidase, is encoded by *B. anthracis* γ phage, which is used by the U.S. Centers for Disease Control and Prevention (CDC) in Atlanta for the identification of *B. anthracis*. It was found that the enzyme could lyse only *B. anthracis* (of all the 14 isolates tested, some were capsulated) and one *Bacillus cereus* strain (RSVF1) closely related to *B. anthracis*, while several other Gram-positive and Gram-negative bacterial species were resistant. Aside from vegetative bacterial cells, germinating *B. anthracis* spores were also susceptible to PlyG, whereas in the dormant state they were resistant. In a murine model of *B. anthracis* infection, one i.p. injection of PlyG 15 min after inoculation with a lethal dose of RSVF1 cells rescued (depending on the dose of enzyme) 68.4% or 76.9% of mice. PlyG can also be used for the specific identification of *B. anthracis* spores. In this assay, spores exposed to a germinant and lysin release adenosine triphosphate (ATP) that can be measured by means of a handheld luminometer as light emitted in the presence of luciferin/luciferase. This method was shown to be very rapid and allowed for the identification of *B. anthracis* spores within 10–60 min depending on the number of spores.

The second lysin, derived from a *B. anthracis* λ Ba02 prophage, is PlyL amidase (Low et al. 2005). This enzyme is interesting in that it displays more potent activity against *B. cereus* than *B. anthracis*. Remarkably, the lytic activity of the full-length PlyL is lower than that of its C-truncated form, most likely due to some inhibitory effects of

the C-terminal domain on the N-terminal enzymatic domain, which could be relieved upon the enzyme's binding to the bacterial cell wall. Interestingly, the removal of the C-terminal domain of another lysin, Ply21 from the *B. cereus* phage TP21, had opposite effects on its capability to lyse different bacterial species. While the C-truncated enzymatic domain of Ply21 displayed higher lytic activity against *Bacillus subtilis*, its ability to lyse *B. cereus* was lower than that of the full-length enzyme (Loessner et al. 1997).

The third lysin capable of lysing *B. anthracis* is PlyPH, an enzyme of putative bacteriophage origin (Yoong et al. 2006). The antibacterial range of this enzyme, like that of PlyG, is practically restricted to *B. anthracis*. The most interesting feature of PlyPH is that it retains lytic activity over a broad range of pH values. While its maximum activity was noted between pH values of 4.5 and 8.0, partial activity was maintained between pH 4.0 and 10.5. PlyPH was also shown to display substantial antibacterial activity in a murine model of peritonitis.

The last enzyme that was reported as a potential means of preventing or treating anthrax is PlyB, a putative muramidase encoded by the BcpI bacteriophage (Porter et al. 2007). The lytic activity of PlyP against a *B. anthracis*-like strain was comparable with that of PlyG. Unlike PlyL, PlyP exhibits its maximum activity in its full-length form, while the C-truncated form is substantially less efficient.

2.1.6. Lysins Specific to Other Bacterial Species Other lysins that might find use as antibacterial agents are Ply118, Ply500, Ply3626, and PlyV12. Ply118 and Ply500 are L-alanyl-D-glutamate peptidases encoded by *Listeria monocytogenes* phages A118 and A500, respectively (Loessner et al. 2002). The antibacterial range of both enzymes is essentially restricted to the genus *Listeria*, and they are not capable of lysing other Gram-positive or Gram-negative bacteria with the exception of *Bacillus megaterium*. It was also shown that they can lyse bacteria only in their full-length forms, while the removal of either of the two major domains resulted in a loss of lytic capacity.

Ply3626 is a putative amidase encoded by *Clostridium perfringens* bacteriophage Ø3626 (Zimmer et al. 2002). It was shown that the antibacterial range of this enzyme is restricted to the species *C. perfringens*. Interestingly, the lytic spectrum of the enzyme is much broader than that of Ø3626 phage. While the phage can infect and kill only 22% of *C. perfringens* strains, the enzyme was capable of lysing all of the 48 strains tested.

PlyV12 is a putative amidase encoded by *E. faecalis* bacteriophage Φ1 (Yoong et al. 2004). This lysin is very interesting in that it is one of

the very few phage lytic enzymes possessing a broad antibacterial range. *In vitro*, PlyV12 was capable of lysing *E. faecalis* and *E. faecium* (all 15 clinical and laboratory strains were tested, including 5 vancomycin-resistant strains) as well as *S. pyogenes* and groups B, C, E, and G streptococci. Moreover, the enzyme displayed weak lytic activity against *S. aureus* and some commensal bacteria.

3. AUTOLYSINS

Another class of lytic enzymes that could be used as enzybiotics are autolysins. These are enzymes encoded by bacteria that are involved in different essential processes of bacterial cells, including cell growth and division, cell wall turnover, bacterial protein secretion, and peptidoglycan maturation (Vollmer et al. 2008). To the best of our knowledge, the first (and hitherto only) autolysin tested as a potential antibacterial agent was LytA amidase, the main autolysin of *S. pneumoniae*. In the first study aimed at evaluating the therapeutic efficacy of LytA, its antibacterial activity was compared with those of Cpl-1 lysin and cefotaxime (Rodriguez-Cerrato et al. 2007). The minimum inhibitory concentration (MIC) values of LytA, Cpl-1, and cefotaxime for a β-lactam-resistant pneumococcal isolate were 16, 32, and 4 μg/mL, respectively. In time-kill experiments, the activities of both enzymes were comparable, and much higher than that of cefotaxime. In a murine model of pneumococcal peritonitis-sepsis, LytA was essentially the most effective of the three studied agents with respect to decreasing bacterial titers in peritoneal fluid and blood.

4. BACTERIOCINS

Bacteriocins are peptides or proteins produced by bacteria to inhibit the growth of other bacteria (Nes et al. 2007). This sets them apart from autolysins, which act on the same bacterial cells in which they were produced. The bacteriocin whose antibacterial activity has been studied most thoroughly both *in vitro* and *in vivo* is lysostaphin.

4.1. Lysostaphin

Lysostaphin is discussed in more detail in Chapter 7. In this section we will present only the most important data on this enzyme and sum up the results of its use as an antibacterial agent.

Lysostaphin is an endopeptidase encoded by *Staphylococcus simulans* biovar *staphylolyticus* that specifically cleaves glycyl-glycyl bonds

in the interpeptide cross-bridges of the staphylococcal peptidoglycan (Thumm and Götz 1997). Lysostaphin is very efficient in lysing *S. aureus* and can kill practically all strains of this species, including MRSA (von Eiff et al. 2003) and strains with reduced susceptibility to vancomycin (Patron et al. 1999). However, its activity against coagulase-negative staphylococci is essentially weaker due to a different amino acid composition of their cross-bridges (Kumar 2008). Aside from planktonic staphylococcal cells, lysostaphin can also specifically eliminate staphylococcal biofilms (Wu et al. 2003).

The first potential medical application of lysostaphin is the elimination of staphylococci colonizing nasal mucous membrane, which, in some clinical settings, may be a starting point for serious infections. In a cotton rat model of *S. aureus* nasal colonization, lysostaphin was shown to be more effective than mupirocin, which is currently the main antibiotic used as a decolonizing agent (Kokai-Kun et al. 2003). Another prophylactic use of lysostaphin might be prevention of catheter colonization by enzyme molecules coating their surface (Shah et al. 2004).

The second major application of lysostaphin can be the treatment of staphylococcal infections, both topical and systemic. So far, the therapeutic effectiveness of lysostaphin has been evaluated in experimental models of bacteremia, endocarditis, neonatal infections, and ocular infections, especially endophthalmitis and keratitis (Patron et al. 1999; Dajcs et al. 2000; Dajcs et al. 2001; Kokai-Kun et al. 2007; Oluola et al. 2007). Essentially, these studies revealed that lysostaphin can efficiently kill bacteria *in vivo* without causing any serious side effects. It is also noteworthy that in some experiments, lysostaphin was found to be more effective than antibiotics (Climo et al. 1998). Importantly, it was shown that specific antibodies do not completely neutralize, but rather moderately reduce, lysostaphin's antibacterial activity *in vivo*, which suggests that the enzyme could exert substantial antibacterial activity even after repeated injection (Climo et al. 1998; Dajcs et al. 2002). It was also found that lysostaphin can exert a synergistic antibacterial activity with other lytic enzymes, cationic antimicrobial peptides, and some antibiotics (Polak et al. 1993; Graham and Coote 2007; Becker et al. 2008). Development of resistance to lysostaphin, at least in some cases, can result in an increase in bacterial sensitivity to antibiotics and a reduction in their fitness and virulence (Kusuma et al. 2007).

4.2. Other Bacteriocins

Another bacteriocin whose antibacterial activity was studied in more detail and which could be used as an antibacterial agent is zoocin A,

produced by *S. equi* ssp. zooepidemicus 4881 (Akesson et al. 2007). It is composed of an N-terminal catalytic domain of putative endopeptidase activity and a C-terminal cell wall-binding domain (Lai et al. 2002). It was shown that zoocin A-susceptible streptococcal species include, aside from *S. equi*, *S. pyogenes*, *Streptococcus mutans*, and *S. gordonii*. It is worth noting that all five *S. pyogenes* strains tested were extremely sensitive to zoocin A (MIC ≤ 31.5 ng/mL). Some other streptococcal species, especially *S. oralis* and *S. rattus*, are not susceptible to the enzyme's lytic activity (Akesson et al. 2007). Interestingly, zoocin A was found to be a penicillin-binding protein (PBP). In this regard, the enzyme was shown to bind penicillin covalently, to possess a weak β-lactamase activity, and to contain motifs typical of other PBPs. Furthermore, incubation of zoocin A with penicillin decreased its enzymatic activity (Heath et al. 2004). Zoocin A was shown to be capable of killing *S. mutans* in a triple-species plaque model (this species is involved in the pathogenesis of dental caries; Simmonds et al. 1995).

5. LYSOZYMES

Lysozymes, or *N*-acetylmuramidases, are hydrolases that specifically cleave the β-1,4 glycosidic linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine in peptidoglycan (Jolles and Jolles 1984). Lysozymes are produced by cells of many different animal species, plants, insects, bacteria, and viruses. Based on their amino acid sequences and structural features, lysozymes are divided into several main subfamilies (Masschalck and Michiels 2003). In the human organism, lysozyme is produced by cells of the immune system (polymorphonuclear granulocytes, monocytes, macrophages) and is found in different biological fluids and tissues, including tears, urine, milk, saliva, liver, cartilage, and skin. However, the best known and most often used lysozyme is hen egg white lysozyme (Jolles and Jolles 1984; Masschalck and Michiels 2003).

Lysozymes are unique enzybiotics in that they exert not only antibacterial activity, but also antiviral, anti-inflammatory, anticancer, and immunomodulatory activities (Sava 1996). They are also the only peptidoglycan hydrolases that have been used on a larger scale in humans for the past several decades.

Although the best known mode of antibacterial action of lysozyme is based on the enzymatic cleavage of peptidoglycan, in fact it can also kill bacteria by some nonenzymatic mechanisms. First, lysozyme, in view of its cationic nature, can activate bacterial autolytic enzymes (autolysins). The second nonenzymatic mechanism by which lysozyme

can kill bacteria is cytoplasmic membrane destabilization resulting from the removal of divalent ions from the membrane surface. In fact, some studies show that nonenzymatic mechanisms may be more important for killing bacteria than the enzymatic cleavage of peptidoglycan (Masschalck and Michiels 2003).

Generally, lysozyme is capable of killing only Gram-positive bacteria, while Gram-negative bacteria are resistant owing to the presence of the outer membrane. However, several exceptions to this rule have been reported, including both lysozyme-resistant Gram-positive bacteria (e.g., some strains of *S. aureus* and *E. faecalis*) and lysozyme-sensitive Gram-negative bacteria (e.g., *Capnocytophaga gingivalis*). It is also worth mentioning that several modifications of the lysozyme molecule have been developed to enable the enzyme to kill Gram-negative bacteria. These are essentially based on coupling lysozyme to molecules facilitating the penetration of the outer membrane (e.g., fatty acids and hydrophobic peptides) (Ibrahim et al. 2002; Masschalck and Michiels 2003).

For the past several decades, lysozyme has been used, often combined with antibiotics, in the prophylaxis and treatment of different bacterial infections, including pharyngitis, tonsillitis, dysentery, and wound infections (Sava 1996). More recently, patents for several lysozyme applications have been applied for or issued (Donovan 2007). These include the use of lysozyme formulated as a gel for topical treatment of wounds, the treatment of acne using different formulations of the enzyme, the prophylaxis of infections due to skin piercing, and the use of aerosolized lysozyme for the treatment of tracheitis, pneumonia, amyglalitis, and faucitis. Another interesting application of lysozyme is the use of its mutant to neutralize the activity of a lysozyme inhibitor produced by *Treponema pallidum*. Lysozyme has also been used as a component of oral health products (e.g., mouthwashes; Tenovuo 2002; Gil-Montoya et al. 2008), taking advantage of its capacity to kill different oral bacteria. A recent study showed that lysozyme can be utilized as a carrier allowing specific delivery of antibiotic molecules to bacterial cells (Hoq et al. 2008).

6. IMPORTANT ASPECTS OF ENZYBIOTIC THERAPY

6.1. Resistance

An important feature of some lytic enzymes, especially lysins, is the low risk of developing resistance. This results likely from the fact that

lysins interact with those components of the cell wall that are necessary for bacterial viability (Borysowski et al. 2006; Fischetti 2008). For instance, the receptor for *S. pneumoniae*-specific lysins is choline, an essential component of the pneumococcal cell wall. In fact, in none of the hitherto conducted studies have any lysin-resistant bacteria been identified (Loeffler et al. 2001). In two separate studies, both *S. pneumoniae* and *B. cereus* failed to develop resistance to lysin even following repeated exposure to low doses of enzyme (the same results were obtained in experiments carried out on solid media and in liquid cultures; Loeffler et al. 2001; Schuch et al. 2002). Furthermore, *B. cereus* remained sensitive to lysin even after exposure to mutagens that rapidly induced mutations resulting in resistance to novobiocin and streptomycin (Schuch et al. 2002). On the other hand, it was found that bacteria can be less susceptible to lysins during the stationary phase, probably owing to some changes in the cell wall composition (Borysowski et al. 2006).

While no cases of bacterial resistance to lysins have been reported as yet, four mechanisms inducing resistance to lysostaphin have been identified (Shaw et al. 2005; Gründling et al. 2006; Kusuma et al. 2007). These are described in more detail in Chapter 7. Interestingly, in some cases the development of resistance to lysostaphin in MRSA results in an increase in susceptibility to methicillin and other antibiotics as well as a reduction in bacterial fitness and virulence (Kusuma et al. 2007).

Moreover, two general mechanisms mediating bacterial resistance to lysozyme have been reported. The first is based on the modifications of some peptidoglycan components (either O-acetylation of *N*-acetylmuramic acid residues by O-acetyltransferase or deacetylation of *N*-acetylglucosamine residues by *N*-acetylglucosamine deacetylase; Vollmer and Tomasz 2000; Bera et al. 2005). Such modified residues restrict the access of lysozyme to its substrate. The other mechanism involves the production of a lysozyme inhibitor (Binks et al. 2005).

6.2. Gram-negative Bacteria

While in Gram-positive bacteria peptidoglycan is easily accessible to recombinant lytic enzymes from outside the cell, in Gram-negative bacteria it is protected by the outer membrane that is impermeable to macromolecules (Vaara 1992). Therefore, Gram-negative bacteria are essentially resistant to lytic enzymes. However, the results of some studies indicate that these bacteria can also be killed by recombinant lytic enzymes

(Masschalck and Michiels 2003; Borysowski et al. 2006). For instance, two lysins were reported to be capable of killing Gram-negative bacteria (Düring et al. 1999; Orito et al. 2004). Moreover, different modifications of enzyme molecules were developed to enable lytic enzymes to penetrate the outer membrane (Ibrahim et al. 2002).

6.3. Immunogenicity

One of the major factors that can decrease the efficacy of protein therapeutics is the induction of a humoral immune response (De Groot and Scott 2007). However, a number of studies have consistently shown that specific antibodies do not completely block, but at most moderately reduce, the antibacterial activity of lytic enzymes (Borysowski et al. 2006; Fischetti 2008). For instance, in a murine model of pneumococcal bacteremia it was shown that a second dose of Cpl-1 administered to mice i.p. 10 days after the first dose can also cure mice from infection (Jado et al. 2003). Similar results were obtained by Loeffler et al. (2003), who found that Cpl-1 had comparable efficacy in terms of decreasing bacterial titers in the blood in naive mice and mice that were administered three i.v. doses of the enzyme 4 weeks earlier. In the same study it was shown that preincubation of Cpl-1 with hyperimmune rabbit serum for 10 or 60 min resulted in only a slight decrease in the enzyme's lytic activity *in vitro* (Loeffler et al. 2003). The most likely explanation for these unexpected findings is the very high affinity of lysins to their receptors on the bacterial cell wall (Fischetti 2008). These results are very important because they indicate that the apparent immunogenicity of lysins may not considerably decrease their therapeutic efficacy following repeated systemic administration. There are also data showing that specific antibodies do not completely neutralize, but rather to some extent decrease, the therapeutic efficacy of lysostaphin *in vivo* (Climo et al. 1998; Dajcs et al. 2002).

It is also worth mentioning that the immunogenicity of lytic enzymes can be considerably reduced by coupling enzyme molecules to polyethylene glycol (PEG), as shown for lysostaphin. However, it has not yet been shown whether modified lysostaphin with better pharmacokinetic features indeed has a higher therapeutic efficacy *in vivo* (Walsh et al. 2003).

6.4. Safety

The major mode of the antibacterial action of lytic enzymes relies on the enzymatic cleavage of peptidoglycan, which is an exclusive com-

ponent of bacterial cells. Therefore, lytic enzymes are not likely to adversely affect mammalian cells, at least not directly (Fischetti 2003; Borysowski et al. 2006). To the best of our knowledge, in no study involving the administration of a lytic enzyme to experimental animals have any serious side effects been found. For instance, no signs of toxicity were detected following repeated administration of lysins to mice regardless of the route of administration (Fischetti 2003; Loeffler et al. 2003). However, it is noteworthy that some glycylglycine endopeptidases, especially lysostaphin, were shown to degrade elastin, which has a high content of glycine residues (Park et al. 1995). This suggests that lysostaphin (and perhaps also other peptidases) could cleave other proteins present in mammalian tissues. It remains to be verified whether or not this activity may translate into any side effects of lytic enzymes.

Another important aspect of the safety of enzybiotic therapy is the possibility of the release of different pro-inflammatory components from bacterial cells being lysed. These components include especially endotoxin, peptidoglycan, as well as teichoic and lipoteichoic acids. Theoretically, massive release of these components could lead to septic shock and multiple organ failure (Nau and Eiffert 2002). However, many experimental studies have consistently shown that even massive bacteriolysis during treatment of systemic bacterial infections, including bacteremia, does not result in any serious side effects. For instance, practically no side effects were found following treatment of bacteremic animals with lysins and lysostaphin, which rapidly reduced bacterial titers in blood (Jado et al. 2003; Loeffler et al. 2003; Kokai-Kun et al. 2007).

Moreover, side effects associated with enzybiotic therapy might occur following the massive release of preformed bacterial toxins from the cytoplasm of bacteria during bacteriolysis. In fact, autolysins of some bacterial species may be involved in the pathogenesis of infections in the mechanism based on the release of different toxins. For instance, autolysins of *Clostridium difficile* may be involved in the release of toxin A and toxin B (Dhalluin et al. 2005). This potential effect should also be taken into account in the discussion about the safety of enzybiotic therapy.

7. CONCLUDING REMARKS

Many experimental studies performed both *in vitro* and *in vivo* have shown that enzybiotics constitute highly effective antibacterial agents.

In view of the dramatic and continuing increase in the prevalence of multidrug-resistant bacteria, the most important features of enzybiotics are their novel mode of action and the capability to kill antibiotic-resistant bacteria. Moreover, at least for some lytic enzymes, the risk of developing resistance is relatively lower than for traditional antibiotics. While unmodified enzybiotics essentially lyse only Gram-positive bacteria, some modifications were developed that enable them to also kill Gram-negative bacteria.

The potential medical applications of enzybiotics include different forms of prophylaxis and treatment of bacterial infections. For instance, some lytic enzymes were shown (in animal models) to be very effective in killing bacteria colonizing mucous membranes upon topical administration. These enzymes could be employed as a unique means of prophylaxis based on clearing bacteria that present a potential starting point for infections. Many experimental studies have also shown that lytic enzymes are efficacious in the treatment of systemic infections, including bacteremia, even in immunized animals.

In view of the unique therapeutic capabilities they provide, lytic enzymes definitely deserve a wider attention of the medical community.

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