Lysin Therapy for *Staphylococcus aureus* and Other Bacterial Pathogens

Vincent A. Fischetti

Abstract Lysins are a new and novel class of anti-infectives derived from bacteriophage (or phage). They represent highly evolved enzymes produced to cleave essential bonds in the bacterial cell wall peptidoglycan for phage progeny release. Small quantities of purified recombinant lysin added externally to gram-positive bacteria results in immediate lysis causing log-fold death of the target bacterium. Lysins can eliminate bacteria both systemically and topically, from mucosal surfaces and biofilms, as evidenced by experimental models of sepsis, pneumonia, meningitis, endocarditis, and mucosal decolonization. Furthermore, lysins can act synergistically with antibiotics by resensitizing bacteria to non-susceptible antibiotics. The advantages over antibiotics are their specificity for the pathogen without disturbing the normal flora, the low chance of bacterial resistance, and their ability to kill colonizing pathogens on mucosal surfaces, a capacity previously unavailable. Lysins, therefore, may be a much-needed anti-infective in an age of mounting antibiotic resistance.

Contents

V.A. Fischetti (✉)
Laboratory of Bacterial Pathogenesis and Immunology, Rockefeller University, 1230 York Avenue, New York, NY 10021, USA
e-mail: vaf@rockefeller.edu

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1 Introduction

Staphylococcus aureus are responsible for severe secondary infections in immunocompromised individuals, as well as disease in otherwise healthy individuals; it is the most common cause of human bacterial infections worldwide (Lowy 1998). Besides skin and soft tissue infections (SSTIs), *S. aureus* can cause sepsis, toxic shock syndrome, pneumonia, necrotizing fasciitis, pyomyositis, endocarditis, and impetigo (White and Smith 1963; Wertheim et al. 2005). Unfortunately, many *S. aureus* strains, such as MRSA and vancomycin-resistant *S. aureus* (VRSA), have evolved resistance to one or more antibiotics used as standard therapy. MRSA account for more than 50 % of hospital isolates causing pneumonia and septicemia (Klein et al. 2007) particularly in intensive care units resulting in 30–40 % mortality (Tiemersma et al. 2004; Laupland et al. 2008). While health-care-associated MRSA infect susceptible patients, community-associated MRSA (CA-MRSA) infect healthy individuals (Herold et al. 1998; Centers for Disease Control and Prevention 1999). CA-MRSA strains are more virulent and contagious and are capable of causing more severe diseases (Miller et al. 2005; Li et al. 2009).

The long-term and large-scale use of antibiotics in human and veterinary medicine provides a powerful selective pressure for antibiotic resistance to arise and eventually dominate populations of human pathogenic microorganisms (Andersson and Hughes 2010). Spontaneous resistance to most antibiotics appears with frequencies ranging from $\leq 10^{-8}$–10^{-11} and, through a series of successive mutations, ultimately generates clinically significant resistance which can then be mobilized in an intra- and interspecies manner by genetic elements such as transposons, plasmids, integrons, and genomic islands (Woodford and Ellington 2007). The first reported human case of MRSA was in Boston in 1968 (Barrett et al. 1968) which increased over the subsequent decades. The evolution of multidrug resistance and the international dissemination of epidemic clones compound the problem, highlighting the need for new antimicrobial development strategies.

Interest in phages and phage products as antimicrobials has been recently renewed in order to address the problem of evolving resistance to antibiotics (O'Flaherty et al. 2009). The use of lysins, or bacteriophage-encoded cell wall hydrolases, has received particular attention because of a potent and often species-specific bacteriolytic activity and a notable lack of bacterial resistance to lysin activity (Fenton et al. 2010; Fischetti et al. 2006). In the context of a phage lifecycle inside a bacterial host, lysins are expressed during viral replication and are ultimately used to cleave the peptidoglycan, lyse the bacterium, and release progeny virions. Purified recombinant lysins, on the other hand, can also be potent lytic agents outside the viral context, driving "lysis from without" of target bacteria both in vitro and in experimentally infected animals (Fenton et al. 2010; Schuch et al. 2002a; Cheng et al. 2005; Ahmed et al. 2011). Potentially therapeutic lysins generally have modular structures defined by well-conserved N-terminal peptidoglycan-cleaving domains and more divergent C-terminal cell wall-binding domains (CBD) that can recognize species-specific cell wall glycopolymers.

The largely universal nature of lysin-sensitive cleavage sites in the peptidoglycan, combined with an increasing understanding of roles for cell wall glycopolymers in maintaining cell wall integrity, is cited to explain the absence of resistance to certain lysins (Fischetti et al. 2006; Fischetti 2010).

Phage lysins have been refined by phage over millions of years to lyse bacteria. They target the integrity of the cell wall and are designed to attack one of the four major bonds in the peptidoglycan. With few exceptions (Loessner et al. 1997), lysins do not have signal sequences, so they are not translocated through the cytoplasmic membrane to attack their substrate in the peptidoglycan; this movement is controlled by a second phage gene product in the lytic system, the holin (Wang et al. 2000). During phage development in the infected bacterium, lysin accumulates in the cytoplasm in anticipation of phage maturation. At a genetically specified time, holin molecules are inserted in the cytoplasmic membrane forming patches, ultimately resulting in generalized membrane disruption (Wang et al. 2003), allowing the cytoplasmic lysin to access the peptidoglycan, thereby causing cell lysis and the release of progeny phage (Wang et al. 2000). Scientists have been aware of the lytic activity of phage for nearly a century, and while whole phages have been used to control infection (Matsuzaki et al. 2005), not until recently have lytic enzymes been exploited for bacterial control in vivo (Nelson et al. 2001; Schuch et al. 2002a; Loeffler et al. 2003). Previous data indicate that lysins work only with gram-positive bacteria, since they are able to make direct contact with the cell wall carbohydrates and peptidoglycan when added externally, whereas the outer membrane of gram-negative bacteria prevents this interaction. However, recently lysins have been identified that are active against certain gram-negative pathogens (Lai et al. 2011; Lood et al. 2015).

2 Mechanism of Action

By thin-section electron microscopy of lsin-treated bacteria, it appears that lysins exert their lethal effects by forming holes in the cell wall through peptidoglycan digestion. The high internal pressure of bacterial cells (roughly 10–15 atmospheres for gram positives) is controlled by the highly cross-linked cell wall peptidoglycan (Fig. 1). Any disruption in the wall's integrity will result in the extrusion of the cytoplasmic membrane and ultimate hypotonic lysis (Fig. 2). Catalytically, a single enzyme molecule should be sufficient to cleave an adequate number of bonds to kill an organism; however, it is uncertain at this time whether this theoretical limit is possible. The reason comes from the work of Loessner (Loessner et al. 2002), showing that a listeria phage enzyme had a binding affinity approaching that of an IgG molecule for its substrate, suggesting that phage enzymes, like cellulases (Jervis et al. 1997) are one-use enzymes, likely requiring several molecules attacking a local region to sufficiently weaken the cell wall.

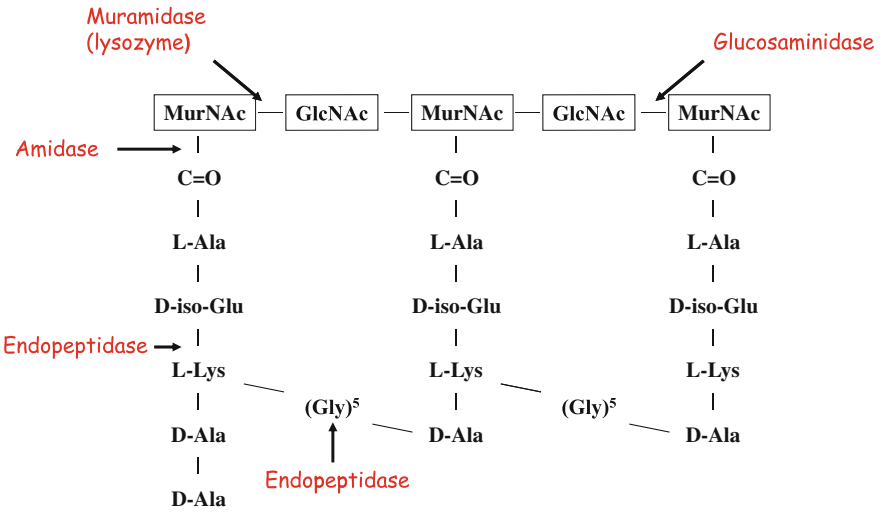

Fig. 1 Diagram of the staphylococcal cell wall peptidoglycan and bonds cleaved by phage lysins. Repeating units of MurNAc (N-acetylmuramic acid) and GlcNAc (N-acetylglucosamine) compose the glycan strands that are linked to a stem peptide through an amide bond to the MurNAc. L-Ala (L-alanine), D-iso-Glu (D-iso-glutamic acid), L-Lys (L-lysine), D-Ala (D-alanine). Stem peptides are then cross-linked through a pentaglycine (in the case of *S. aureus*) to adjacent stem peptides forming a tight stable net around the bacterium. Lysins cleave the major bonds: amidase (N-acetylmuramoyl L-alanine amidase), muramidase (N-acetylmuramidase), glucosamidase (N-acetylglucosaminidase), and endopeptidase

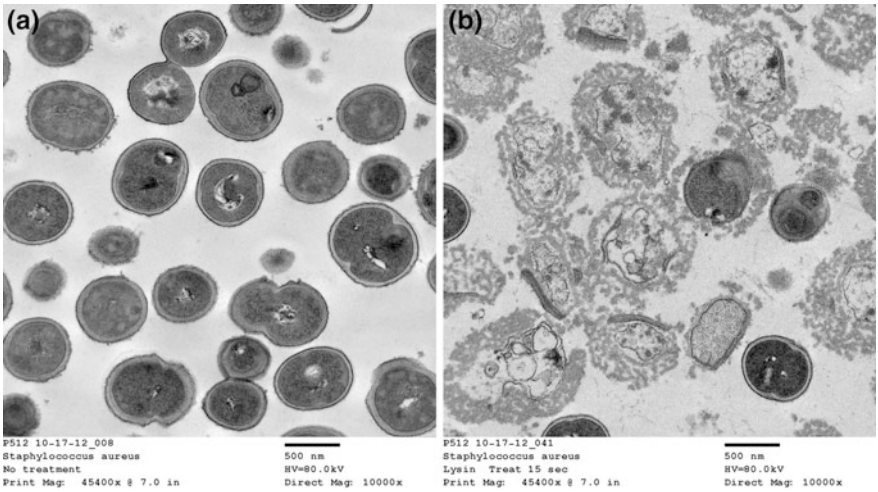

Fig. 2 Thin-section electron micrographs of *S. aureus* before (**a**) and after (**b**) treatment with phage lysin PlySs2 for 15 s

3 Lysin Efficacy

In general, lysins only kill the species (or subspecies) of bacteria from which they were produced. For instance, enzymes produced from streptococcal phage kill certain streptococci, and enzymes produced by pneumococcal phage kill pneumococci (Loeffler et al. 2001; Nelson et al. 2001). Specifically, a lysin from a group C streptococcal phage (PlyC) will kill group C streptococci, as well as groups A and E streptococci (human pathogens), the bovine pathogen *Streptococcus uberis,* the horse pathogen, *Streptococcus equi*, and the bovine pathogen *S. uberis,* but essentially no effect on streptococci normally found in the oral cavity of humans and other gram-positive bacteria. Similar results are seen with a pneumococcal-specific lysin (Cpl-1) (Loeffler et al. 2001). The most specific lysin reported to date is PlyG directed to *Bacillus anthracis* (Schuch et al. 2002a). This enzyme binds to a neutral polysaccharide found only in the cell wall of *B. anthracis* and rare "transition strains" of *B. anthracis*/*Bacillus cereus* (Schuch et al. 2013). Thus, unlike antibiotics, which are usually broad spectrum and kill many different bacteria found in the human body, some of which are beneficial, lysins may be identified which kill only the disease organism with little to no effect on the normal human bacterial flora. In some cases, however, phage enzymes may be identified with broad lytic activity. One such lysin from an enterococcal phage has recently been reported to not only kill enterococci but a number of other gram-positive pathogens such as *Streptococcus pyogen*es, group B streptococci, and *S. aureus* (Yoong et al. 2004). However, the broadest lysin identified is from a bacteriophage that infects *Streptococcus suis* (a pig pathogen). This enzyme (PlySs2) not only kills *S. suis* but other gram-positive pathogens (MRSA, vancomycin-intermediate *S. aureus* (VISA), Listeria, *Staphylococcus simulans, Staphylococcus epidermidis, S. equi, Streptococcus agalactiae*, *S. pyogenes, Streptococcus sanguinis, group G streptococci*, group E streptococci, and *Streptococcus pneumoniae*) (Gilmer et al. 2013).

4 *Staphylococcus aureus*-Specific Lysins

An important lysin with respect to infection control would be lysins directed to *S. aureus* (Sonstein et al. 1971; Clyne et al. 1992; O'Flaherty et al. 2005; Rashel et al. 2007; Manoharadas et al. 2009; Gilmer et al. 2013). In an earlier publication (Rashel et al. 2007), a staphylococcal enzyme was described that could be easily produced recombinantly and had a significant lethal effect on methicillin-resistant *S. aureus* (MRSA) both in vitro and in a mouse model. In the animal experiments, the authors show that the enzymes may be used to decolonize staphylococci from the nose of the mice as well as protect the animals from an intraperitoneal challenge with MRSA. However, in the latter experiments, the best protection was observed if the lysin was added up to 30 min after the MRSA. To help overcome insolubility

problems inherent in many staphylococcal-specific lysins, a chimeric enzyme was produced from two different *S. aureus*-specific lysins; however, though soluble, the activity did not show log-fold drop in viability or efficacy in animal models (Manoharadas et al. 2009). Subsequently, a chimeric lysin (ClyS) was produced from two staphylococcal-specific lysins (PhiNM3 and Twort). Purified ClyS efficiently lysed MRSA, VISA, and methicillin sensitive (MSSA) strains of *S. aureus* by >2-logs in vitro. In a mouse nasal decolonization model, a 2-log reduction in the viability of MRSA cells was seen 1 h following a single treatment with ClyS and one intraperitoneal dose of ClyS also protected against death by MRSA in a mouse bacteremia model. More recently, PlySs2 (described above as one of the broadest acting lysins) (Gilmer et al. 2013) was used to control MRSA. PlySs2 at 128 μg/ml in vitro reduced MRSA growth by 5-logs within 1 h and exhibited an MIC of 16 μg/ml for MRSA. A single, 2-mg dose of PlySs2 protected 92 % of the mice in a bacteremia model of mixed MRSA and *S. pyogenes* infection.

5 Synergy

Because of their action on the cell wall, when lysins are used in combination with antibiotics, they tend to work synergistically (Loeffler and Fischetti 2003; Djurkovic et al. 2005; Rashel et al. 2007; Daniel et al. 2010; Schuch et al. 2014; Schmelcher et al. 2015) (Fig. 3). For example, when the pneumococcal lysin Cpl-1 was used in combination with gentamicin, increased synergistic killing of

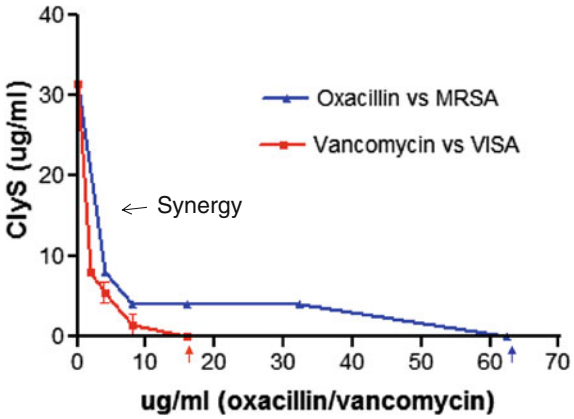

Fig. 3 Results of an isobologram with the staphylococcal-specific lysin (ClyS) and an oxacillin-resistant MRSA strain and a vancomycin-resistant VISA strain of staphylococci. The results show a typical synergistic curve for both organisms, showing that small (non-lytic) amounts of the ClyS lysin will increase the sensitivity of the resistant staphylococci to the respective antibiotics

pneumococci was observed with decreasing penicillin MIC (Djurkovic et al. 2005). Synergy was also observed with a staphylococcal-specific enzyme and glycopeptide antibiotics (Rashel et al. 2007).

In a recent report of a lysin being developed as a therapeutic by Contrafect Corporation (Schuch et al. 2014) (Contrafect.com), a lysin (CF-301) was used in combination with vancomycin or daptomycin to significantly increase the survival of mice with staphylococcal-induced bacteremia. The lysin at sublytic doses apparently weakens the bonds in the peptidoglycan allowing more efficient uptake of the antibiotics. Thus, the right combination of lysin and antibiotic could help in the control of antibiotic-resistant bacteria as well as reinstate the use of certain antibiotics for which resistance has been established.

6 Biofilms

Most bacteria can form biofilms, which are composed of bacterial cells adherent to a substrate and to each other. These adherent cells are usually embedded within a matrix of extracellular slimy material composed of DNA, protein, and polysaccharides secreted and released by the cells. Biofilms are found on living or non-living surfaces and are prevalent wherever bacteria are found. By its very nature, bacteria imbedded within biofilms are more difficult to kill with conventional antibiotics. Because bacteria are part of the biofilm matrix, the lytic action of lysins on the bacterial component of the matrix has a destabilizing effect on these structures. CF-301, the staphylococcal-specific lysin, being developed by Contrafect, was shown to be quite effective against staphylococcal biofilms produced for 24 h on polystyrene dishes and subsequently treated with CF-301. Following treatments for 2 or 4 h, residual biofilms were completely removed by 2 h, whereas standard of care antibiotics at 1000-fold higher concentration failed to remove the biomass even after 4 h of treatment (Schuch et al. 2014). These results could explain earlier studies where a pneumococcal lysin was used to successfully remove pneumococci from the heart valves in a rat endocarditis model (Entenza et al. 2005). By definition, endocarditis is a biofilm of organisms on heart valves, which is difficult to treat and has a high fatality rate, particularly in the case of MRSA endocarditis (Huang et al. 2008). In some cases, surgical intervention is required to remove the biofilm. Lysin treatment, in combination with standard of care antibiotics, could prove more effective than antibiotics alone.

7 Effects of Antibodies

The pharmacokinetics of lysins like other foreign proteins delivered systemically to animals is about 20 min (Loeffler and Fischetti 2003). Thus, if lysins are to be used systemically, they may need to be modified to extend their half-life, or they may

need to be delivered frequently or by IV infusion. However, because lysins work rapidly, more rapidly than antibiotics, perhaps one or two doses may be sufficient. Since lysins are proteins, a concern regarding their use is the development of neutralizing antibodies, which could reduce the in vivo levels and activity of the enzyme during treatment. When rabbit hyperimmune serum raised against the pneumococcal-specific enzyme Cpl-1 was assayed for its effect on lytic activity, it did not block the activity of Cpl-1 (Loeffler et al. 2003). When similar in vitro experiments were performed with antibodies directed to a *B. anthracis*- and an *S. pyogenes*-specific enzyme, similar results were obtained (Fischetti, unpublished data). These results were also verified with a staphylococcal-specific lysin (Rashel et al. 2007).

To test the relevance of this result in vivo, mice that received three intravenous doses of the Cpl-1 enzyme tested positive for IgG against Cpl-1 in 5 of 6 cases with low but measurable titers of about 1:10. Vaccinated and naïve control mice were then challenged intravenously with pneumococci and then treated by the same route with 200 μg Cpl-1 after 10 h. Within a minute, the treatment reduced the pneumococcal titer in the blood of Cpl-1-immunized mice to the same degree as the naive mice, supporting the in vitro data that antibody to lysins has little to no neutralizing effect. A similar experiment by Rashel with a staphylococcal enzyme (Rashel et al. 2007) showed the same result and that animals injected with lysin multiple times exhibited no adverse events.

This unexpected effect may be partially explained if the binding affinity of the enzyme for its substrate in the bacterial cell wall is higher than the antibody's affinity for the enzyme. This is supported by the results of Loessner (Loessner et al. 2002), showing that the cell wall-binding domain of a listeria-specific phage enzyme binds to its wall substrate at nanomolar affinities. However, while this may explain the inability of the antibody to neutralize the binding domain, it does not explain why antibodies to the catalytic domain do not neutralize. Nevertheless, these results are encouraging since it suggests that such enzymes may be used repeatedly in certain situations to control infecting or colonizing disease bacteria.

8 Bacterial Resistance to Lysins

Exposure of bacteria grown on agar plates to low concentrations of lysin did not lead to the recovery of resistant strains even after over 40 cycles. Organisms in colonies isolated at the periphery of a clear lytic zone created by a 10 μl drop of dilute lysin always resulted in enzyme-sensitive bacteria. Enzyme-resistant bacteria could also not be identified after >10 cycles of bacterial exposure to low concentrations of lysin (from 5–20 units) in liquid culture (Loeffler et al. 2001). These results may be explained by the fact that the cell wall receptor for the pneumococcal lysin is choline (Garcia et al. 1983), a molecule that is essential for pneumococcal viability, and the receptor for PlyG, the lysin against *B. anthracis,* is directed against a neutral wall polysaccharide that is essential for *B. anthracis* survival

Table 1 List of pathogens to which phage lysins have been developed

Organisms for which lysins have been developed:	
S. pyogenes	*S. uberis*
S. pneumoniae	*S. equi*
B anthracis	*B. cereus*
Gr. B streptococcus	*B. thuringiensis*
E faecalis/E faecium	*B. megaterium*
S. aureus	Gr. G streptococci
C. perfringens	*A. baumannii*
C. difficile	*S. suis*
P. acnes	

(Schuch et al. 2013). While not yet proven, it is possible that during a phage's association with bacteria over the millennia, to avoid becoming trapped inside the host, the binding domain of their lytic enzymes evolved to target unique and essential molecules in the cell wall, making resistance to these enzymes a rare event.

9 Conclusion

Lysins are a new therapeutic to control a number of bacterial pathogens (Table 1). For the first time, we may be able to specifically kill pathogens on mucous membranes without affecting the surrounding normal flora, thus reducing a significant pathogen reservoir in the population. Since this capability has not been previously available, its acceptance may not be immediate. Nevertheless, such as vaccines, we should be striving to develop methods to prevent rather than treat infection. Whenever there is a need to kill bacteria, and contact can be made with the organism, lysins may be freely utilized. Such enzymes will be of direct benefit in environments where antibiotic-resistant gram-positive pathogens are a serious problem, such as hospitals, day care centers, and nursing homes. The lysins isolated thus far are remarkably heat stable (up to 60 °C) and are relatively easy to produce in a purified state and in large quantities, making them amenable to these applications. The challenge for the future is to use this basic strategy and improve upon it, as was the case for second- and third-generation antibiotics. Protein engineering, domain swapping, and gene shuffling all could lead to better lytic enzymes to control bacterial pathogens in a variety of environments. Since it is estimated that there are 10^{31} phage on earth, the potential to identify new lytic enzymes is enormous. Perhaps, someday phage lytic enzymes will be an essential component in our armamentarium against pathogenic bacteria.

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