Phage Lysins: Novel Alternative to Antibiotics

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1 Background

Viruses that specifically infect bacteria are called bacteriophage (or phage). After replicating inside its bacterial host, the phage is faced with a problem; it needs to efficiently exit the bacterium to disseminate its progeny to begin a new cycle. To solve this, double-stranded deoxyribonucleic acid (DNA) phages have evolved a lytic system to weaken the bacterial cell wall resulting in hypotonic bacterial lysis or "lysis from within."

Phage lytic enzymes or lysins are highly efficient molecules that have been refined over millions of years of evolution to effectively release it progeny phage. These enzymes target the integrity of the cell wall, and are designed to attack one of the five 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 tightly 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 a hole through localized membrane disruption (Wang et al. 2003). The cytoplasmic lysin is now able to access the peptidoglycan to cleave specific bonds, thereby causing immediate cell lysis as a result of internal turgor

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pressure (which in the gram-positive organism could be as much as 15–25 atm), and the release of progeny phage (Wang et al. 2000). In contrast to large DNA phage, small ribonucleic acid (RNA) and DNA phages use a different release strategy. They call upon a phage-encoded protein to interfere with bacterial host enzymes responsible for peptidoglycan biosynthesis (Bernhardt et al. 2001; Young et al. 2000) resulting in misassembled cell walls and ultimate lysis. Scientists have been aware of the lytic activity of phage for nearly a century, and while whole phage have been used to control infection (Matsuzaki et al. 2005), not until recently have lytic enzymes been exploited for bacterial control in vivo (Fischetti 2018; Loeffler et al. 2003; Nelson et al. 2001; Schuch et al. 2002). One of the main reasons that such an approach is only now even being seriously considered, is the sharp increase in antibiotic resistance among pathogenic bacteria. Current data indicate that lysins work best against gram-positive bacteria, since they are able to make direct contact with the cell wall carbohydrates for binding and peptidoglycan cleavage when added externally, whereas the outer membrane of gram-negative bacteria limits this direct interaction. Recently, however, lysins which are able to both disrupt the outer membrane of gram-negative bacteria and cleave the peptidoglycan have been identified (Lai et al. 2011; Lood et al. 2015). Lysins, termed artilysins, have also been engineered to penetrate the outer membrane to kill gram-negative bacteria (Briers et al. 2014). However, in nearly all cases, gram-negative lysins have low or no activity in the presence of serum.

Bacterial lysis can also occur through the addition of an external agent resulting in what is termed "lysis from without." This lytic event can arise either through the exposure of the bacteria to a high multiplicity of infection (MOI) of phage (Abedon 2011; Delbruck 1940) or the exposure of the bacteria to phage-derived or other wall-degrading lysins. These events do not occur to any great extent if at all in nature, but are the result of phage or lysin manipulation in the laboratory. Both events utilize enzymes that cleave the peptidoglycan; in the case of whole phage, lytic enzymes are part of the phage tail structure used to locally degrade the peptidoglycan of either gram-positive or gram-negative bacteria, allowing entry of phage DNA (or RNA in the case of RNA phage) to the cell. Lysis through lytic enzymes may occur in nature if the lysins used by the phage to release progeny happen to interact with sensitive bacteria in the vicinity of the lysing bacteria. But this would be a rare event, since lysins have evolved binding domains to retain the free lysin on the cell wall debris of the lysing bacteria. In general, lysis from without is more likely to occur with gram-positive bacteria since their cell wall peptidoglycan is more directly exposed on the cell surface than gram-negative bacteria, that are protected by an outer membrane. Because both lytic events are rapid and efficient, the power of these enzymes could be harnessed to control bacterial pathogens.

Most human infections (viral or bacterial) begin at a mucous membrane site (upper and lower respiratory, intestinal, urogenital, and ocular). Furthermore, these human mucous membranes are the reservoir (and sometimes the only reservoir) for many pathogenic bacteria found in the environment (i.e., pneumococci, staphylococci, streptococci, hemophilus) some of which are resistant to current antibiotics. In most instances, it is this mucosal reservoir that is the focus of infection in the population

(Coello et al. 1994; de Lencastre et al. 1999; Eiff et al. 2001). To date, except for polysporin and mupirocin ointments, which are the most widely used topically, there are no anti-infectives that are designed to control colonizing pathogenic bacteria on mucous membranes (Hudson and Hay 1976); we usually must wait for infection to occur before treating. Because of the fear of increasing the resistance problem, antibiotics are not indicated to control the carrier state of disease bacteria. It is acknowledged, however, that by reducing or eliminating this human reservoir of pathogens in the community and controlled environments (i.e., hospitals and nursing homes), the incidence of disease will be markedly reduced (Eiff et al. 2001; Hudson and Hay 1976). Toward this goal, an effective use for lysins is to prevent infection by safely and specifically destroying disease bacteria on mucous membranes. For example, based on extensive animal results, lysins specific for *S. pneumoniae* (Loeffler et al. 2001), *S. pyogenes* (Nelson et al. 2001), and *S. aureus* (Gilmer et al. 2013) may be used nasally and/or orally to control these organisms in the community as well as in nursing homes and hospitals to prevent or markedly reduce serious infections caused by these colonizing bacteria.

Like naturally occurring antibiotics, which are used by bacteria to control the organisms around them in the soil environment, phage lysins are the culmination of millions of years of evolution by the bacteriophage in their association with bacteria. Specific lysins have now been identified and purified that are able to kill specific gram-positive bacteria seconds after contact (Loeffler et al. 2001; Nelson et al. 2001). For example, nanogram quantities of lysin could reduce 10^7 *S. pyogenes* by >6-log minutes after enzyme addition. No known biological compounds, except chemical agents, kill bacteria this quickly. Because of their highly effective activity against bacteria for the control of disease, the term "enzybiotics" was coined (Nelson et al. 2001) to describe these novel anti-infectives.

2 Structural Characteristics of Gram-Positive Lysins

Lysins from DNA phage that infect gram-positive bacteria are generally between 25 and 40 kDa in size except the PlyC lysin from the C1 phage of group C streptococci, which is 114 kDa. PlyC is unique because it is composed of two separate gene products, PlyCA and PlyCB. Based on biochemical and biophysical studies, the catalytically active PlyC holoenzyme is composed of eight PlyCB subunits for each PlyCA (Nelson et al. 2003). The eight PlyCB subunits spontaneously form a donut-like structure onto which the two PlyCA catalytic domains are non-covalently attached (McGowan et al. 2012). A feature of all other gram-positive phage lysins is their two-domain structure (Fig. 1). With rare exceptions (Diaz et al. 1990; Garcia et al. 1990), the N-terminal domain contains the catalytic activity of the enzyme. This activity may be either: i) an endo-β-N-acetylglucosaminidase or N-acetylmuramidase (lysozymes), both of which act on the glycan moiety of the wall peptidoglycan, ii) an endopeptidase which cleaves the stem peptide moiety, iii) an N-acetylmuramoyl-L-alanine amidase (or amidase), which hydrolyzes the amide bond connecting the glycan strand and peptide moieties (Loessner 2005; Young 1992), or an

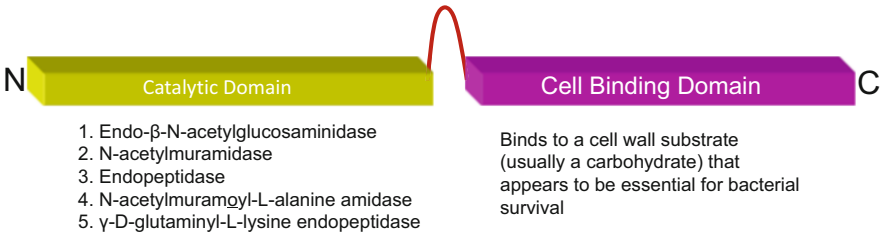

Fig. 1 Basic structure of phage lysins. In general, lysins range between 25 and 40 kDa in size and have a domain structure. The N-terminal domain is invariably the catalytic domain which cleaves one of the five major bonds in the peptidoglycan, and the C-terminal domain binds to a carbohydrate determinant in the cell wall

endopeptidase that specifically cleaves the cross-bridge. Recently a phage lysin with γ-D-glutaminyl-L-lysine endopeptidase activity has also been reported (Pritchard et al. 2007), but these enzymes are not as prevalent as the others. In some cases, particularly in staphylococcal phage and some streptococcal species of phage, two and even three different catalytic domains may be linked to a single binding domain (Cheng et al. 2005; Navarre et al. 1999). The C-terminal cell-binding domain (termed the CBD) on the other hand binds to a specific substrate (usually carbohydrate) found in the cell wall of the host bacterium (Garcia et al. 1988; Lopez et al. 1992, 1997). Efficient cleavage generally requires that the binding domain bind to its cell wall substrate, offering some degree of specificity to the enzyme since these binding substrates are only found in enzyme-sensitive bacteria. However, catalytic domains alone are able to cleave the peptidoglycan in some cases.

The first complete crystal structure for the free and choline-bound states of the Cpl-1 lytic enzyme, which lyses pneumococci, has been published (Hermoso et al. 2003). As suspected, the data suggest that choline recognition by the choline-binding domain of Cpl-1 may allow the catalytic domain to be properly oriented for efficient cleavage. An interesting feature of this lysin is its hairpin conformation suggesting that the two domains interact with each other prior to the interaction of the binding domain with its substrate in the bacterial cell wall (in this case choline). A second lysin, PlyL, encoded by a lysogen in the *Bacillus anthracis* genome, and a third, Ply21, from *B. cereus* phage TP21 have similar characteristics (Low et al. 2005). Their data suggest that the structure of PlyL and Ply21 are also in a hairpin conformation where the C-terminal domain blocks the activity of the catalytic domain through intramolecular interactions that are reversed when the C-terminal domain binds to its substrate in the cell wall freeing the catalytic domain to cleave its substrate. This suggests that lysins are autoregulated so that they are unable to interact with substrates in the cytoplasm that are precursors for cell wall assembly, but function only when they pass through the hole in the membrane to their peptidoglycan substrate. Such an inactivated enzyme would also be unable to kill potential host bacteria nearby if released from a lysed organism, thus preventing its competition with released phage.

When the sequences between lytic enzymes of the same enzyme class (i.e., amidases) are compared, high sequence homology is seen within the N-terminal catalytic region and very little homology in the C-terminal cell-binding region, suggesting that lysins are each tailored for a specific species. It seemed counterintuitive that the phage would design a lysin that was uniquely lethal for its host organism; however, as we learned more about how these enzymes and how they function, a possible reason for this specificity became apparent. As mentioned above, because of their specificity, enzymes that spill after cell lysis have a good chance of killing potential bacterial hosts in the vicinity of the released phage progeny therefore competing for phage survival. Thus, in addition to autoregulating the lysin's activity before contacting its substrate in the peptidoglycan, we believe that the gram-positive lysins have evolved binding domains that bind to their cell wall substrate at a high affinity (Loessner et al. 2002) to limit the release of free enzyme after lysis, so the enzyme remains bound to cell wall debris. This binding substrate in the cell wall appears to be unique with certain species. This does not appear to be the case for lysins produced by gram-negative phage. Thus, in two ways gram-positive phages protect the activity and release of active lysins to prevent competition with free phage.

Because of their domain structure, it seemed plausible that different enzyme domains could be swapped resulting in lysins with different bacterial and catalytic specificities. This was actually accomplished by early detailed studies of Garcia and colleagues (Garcia et al. 1990; Weiss et al. 1999), in which the catalytic domains of lytic enzymes from *S. pneumoniae* phage could be swapped resulting in a new enzyme having the same binding domain for pneumococci, but able to cleave a different bond in the peptidoglycan. In addition, DNA mutagenesis has been used to create lysins with higher antibacterial activity (Cheng and Fischetti 2007). These manipulations allow for enormous potential in creating designer enzymes with high specificity and equally high cleavage potential. In recent years, this idea has been capitalized upon and lysins have been engineered to achieve certain characteristics not present in native lysins (Daniel et al. 2010; Dong et al. 2015; Donovan et al. 2006). For a more complete review of the subject, please see Yang et al. (2014).

3 Structural Characteristics of Gram-Negative Lysins

At this time, only a few lysins from gram-negative phage have been characterized (Briers et al. 2011; Huang et al. 2014; Junn et al. 2005; Lai et al. 2011; Larpin et al. 2018; Lood et al. 2015). One would think that since phage lytic enzymes function from the inside to access and cleave the peptidoglycan, that lysins from both gram-positive and gram-negative bacteria would be the same, but they are largely different. Generally speaking most gram-negative lysins are lysozymes, i.e., muramidases or N-acetylmuramide glycanhydrolase without true binding domains as see with gram-positive lysins. The complexity of gram-negative lysins became apparent when Lood et al. (2015) combined the induced phage from several strains of *A. baumannii*, shotgun cloned their DNA, and determined which clones exhibited lytic activity.

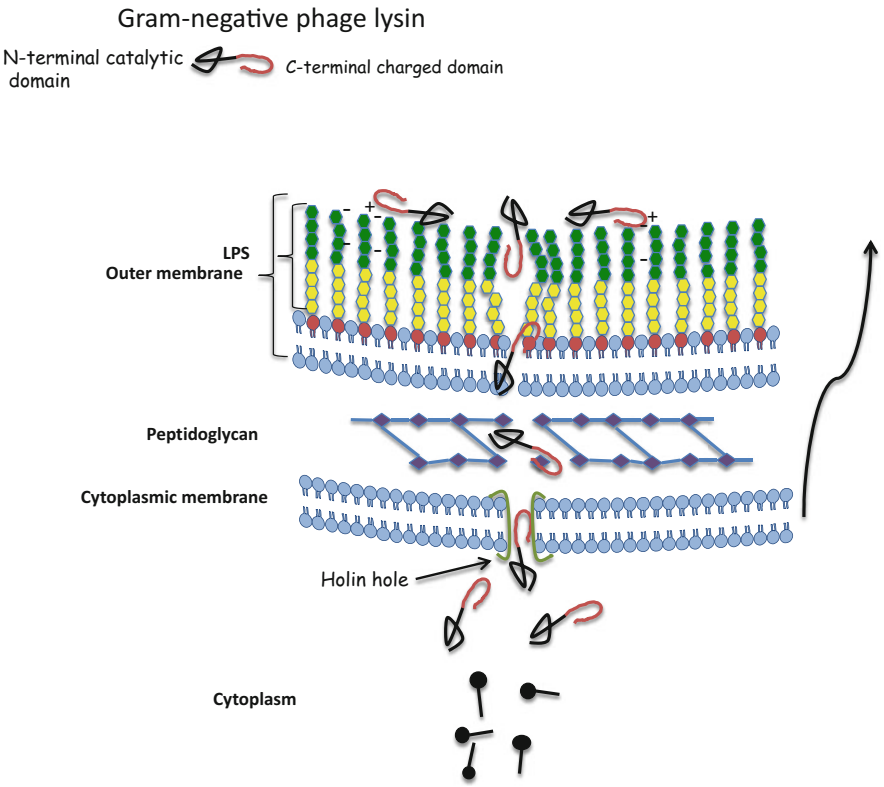

Fig. 2 Proposed model of the action of gram-negative lysins. Based on current data (Lood et al. 2015; Thandar et al. 2016), many gram-negative lysins have a lysozyme catalytic domain and a positively charged domain at the C-terminus. In this model, the two domains are represented in black and orange, respectively. As the lysin passes through the holin-induced hole in the cytoplasmic membrane, it interacts with the peptidoglycan, thereby cleaving the glycosidic bonds with the lysozyme domain. The lysin then interacts with the outer membrane where the positively charged domain destabilizes this region resulting in phage release. The positively charged domain would continue to interact with the negatively charged LPS preventing release of the lysin in the environment which could affect nearby bacterial hosts

When the inserts of several lytic clones were sequenced they found that the lysins fell into three distinct structural groups: (1) a TIGR02594 domain, (2) a catalytic domain and a binding domain like gram-positive lysins, and (3) a lysozyme domain. Both the TIGR02594 and lysozyme domains were flanked on either both or one side with a short positively charged domain, not previously reported. Since gram-negative bacteria have a lower internal turgor pressure (~3–5 atm) than their gram-positive counterparts (~15–25 atm), there may not be sufficient pressure to disrupt the integrity of the outer membrane after the bonds in the peptidoglycan have been cleaved. Thus, data suggests that the lysin's structural organization functions to both cleave the peptidoglycan with its enzymatic domain and disrupt the outer

membrane with the charged domain, resulting in more efficient lysis and phage release (Thandar et al. 2016) (Fig. 2).

4 Mechanism of Action

When examined by thin section electron microscopy, it seems obvious that lysins exert their lethal effects by forming holes in the cell wall through peptidoglycan digestion. The high internal turgor pressure of gram-positive bacterial cells is controlled by the highly cross-linked and thick peptidoglycan. Any disruption in the wall's integrity would result in the extrusion of the cytoplasmic membrane and ultimate hypotonic lysis (Fig. 3). 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 wall substrate. This suggests that each lysin molecule could be a single-use enzyme, requiring several enzymes to effectively cleave enough bonds in a localized area of the cell to cause a lytic event. It should be noted that lysins from gram-positive phage closely resemble fungal cellulases, which are similarly constructed with both cell

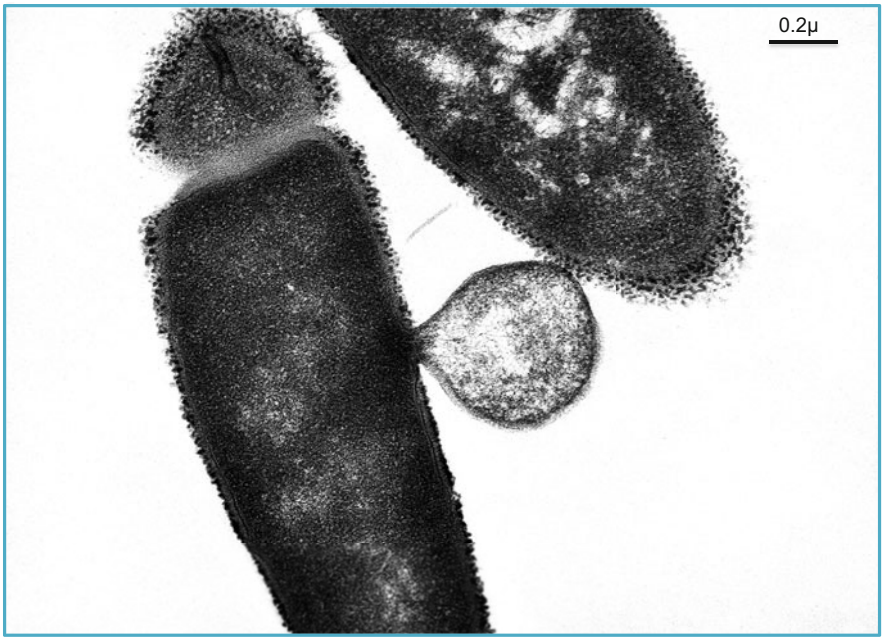

Fig. 3 Electron microscopy of lysin treated bacilli. One minute after treatment of *B. cereus* with lysin, membrane extrusion is observed prior to lysis and ultimate death of the bacterium

wall binding and catalytic domains joined by a flexible linker. It is unknown at this time whether lysins, like cellulases, use their binding domains to "bind and slide" across the wall peptidoglycan as they cleave (Jervis et al. 1997; Payne et al. 2015) or are simply enzymes that stay substrate-bound and are only able to cleave adjacent bonds, i.e., "one use enzymes."

5 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 the C1 streptococcal phage (PlyC) will kill group C streptococci, as well as groups A and E streptococci, the bovine pathogen *S. uberis* and the horse pathogen, *S. equi*, with essentially no effect on other gram-positive bacteria, particularly the commensal streptococci normally found in the oral cavity of humans. Unlike antibiotics, which are usually broad spectrum and kill many different bacteria found in the human body (some of which are beneficial), lysins which kill only the disease organism with little to no effect on the normal human bacterial flora may be identified. One of the most specific lysins reported is the lysin for *B. anthracis* (PlyG); this enzyme only kills *B. anthracis* and rare but unique *B. cereus* strains (Schuch et al. 2002). Another highly specific lysin is a chimeric lysin for staphylococci called ClyS (Daniel et al. 2010). Because ClyS is an endopeptidase that cleaves the peptidoglycan cross-bridge, and only staphylococci have polyglycine in their cross-bridge, this enzyme was shown to have lytic activity on all staphylococci and no other species of bacteria tested (Daniel et al. 2010).

In some cases, however, phage enzymes may be identified with broad lytic activity. For example, an enterococcal phage lysin PlyV12 has been reported to not only kill enterococci but also a number of other gram-positive pathogens such as *S. pyogene*s, group B streptococci, and *Staphylococcus aureus*, making it one of the broadest acting lysins identified (Yoong et al. 2004). However, its activity for these other pathogens was somewhat lower than for enterococci. Similar broad activity was seen for an enzyme called PlySs2 isolated from a *S. suis* phage (Gilmer et al. 2013) with strong activity against a number of other pathogens such as *S. aureus*, *S. suis*, *S. pyogenes*, *group B streptococci*, and *S. pseudintermedius*.

6 Lysin Synergy with Other Lysins and Antibiotics

Several lysins have been identified from pneumococcal bacteriophage which are classified into two groups: amidases and lysozymes. Exposure of pneumococci to either of these enzymes leads to efficient lysis. Both classes of enzymes have very different N-terminal catalytic domains but share a similar C-terminal choline-binding domain. When these enzymes were tested to determine whether their

simultaneous use is competitive or synergistic the results clearly showed that they are synergistic (Loeffler and Fischetti 2003). The same is true for endolysins directed to streptococci responsible for mastitis (Schmelcher et al. 2015). In vivo, the combination of two lysins with different peptidoglycan specificities was found to be more effective in protecting against disease than each of the single enzymes (Jado et al. 2003; Loeffler and Fischetti 2003; Schmelcher et al. 2015). Thus, in addition to more effective killing, the application of two different lysins may significantly retard the emergence of enzyme-resistant mutants.

When the pneumococcal lysin Cpl-1 was used in combination with certain antibiotics, a similar synergistic effect was seen. Cpl-1 and gentamicin were found to be increasingly synergistic in killing pneumococci with a decreasing penicillin MIC, while Cpl-1 and penicillin showed synergy against an extremely penicillin-resistant strain (Djurkovic et al. 2005). Synergy was also observed with staphylococcal-specific lysins and antibiotics with MRSA both in vitro (Fig. 3) (Daniel et al. 2010) and in vivo (Daniel et al. 2010; Rashel et al. 2007). A reason for this effect may be partially explained by the finding that the cell wall of MRSA is less cross-linked than MSSA (Trotonda et al. 2009), allowing small quantities of lysin to have a more dramatic effect on cell wall integrity. Thus, the right combination of enzyme 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.

7 Use of Lysins in Biological Fluids

Based on published findings, lysins from phage that infect gram-positive bacteria generally work in most, if not all bodily fluids: serum (Schmelcher et al. 2015; Witzenrath et al. 2009), saliva (Nelson et al. 2001), intestinal (Wang et al. 2015), and cerebral spinal fluid (Grandgirard et al. 2008). However, it is now clear that lysins from phage that infect gram-negative bacteria as well as Artilysins are unable to function effectively in the presence of serum (Briers et al. 2014; Larpin et al. 2018; Lood et al. 2015), perhaps as a result of binding to serum proteins by the positively charged region at the ends of these molecules (Thandar et al. 2016). Thus, until gram-negative lysins are engineered to avoid these interactions, they may be limited to topical use.

8 Animal Models of Infection

Animal models of mucosal colonization were used to test the capacity of gram-positive lysins to kill organisms on these surfaces, perhaps one of the more important uses for these enzymes. An oral colonization model was developed for *S. pyogenes* (Nelson et al. 2001), a nasal model for pneumococci (Loeffler et al. 2001), and a vaginal model for group B streptococci (Cheng et al. 2005). In all three cases, when the animals were colonized with their respective bacteria and treated with a single dose of lysin, specific for the colonizing organism, these organisms were reduced by

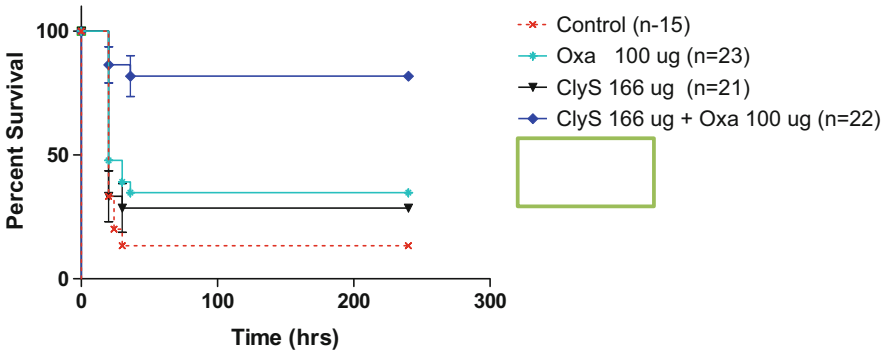

Fig. 4 Synergistic effects of ClyS and oxacillin protected mice from MRSA septicemia-induced death. Mice were intraperitoneally injected with ~5 × 10^5 CFU of MRSA strain MW2 in 5% mucin. Three hours postinfection, mice received an IP injection of a suboptimal concentration of ClyS (166 μg) or 20 mM phosphate buffer along with an IM injection of oxacillin (100 μg) or saline control. Mice were monitored for survival for 10 days, and the results of 5 independent experiments were combined and plotted in a Kaplan Meier survival curve

several logs (and in some cases below the detection limit of the assay) when tested again 2–4 h after lysin treatment. These results lend support to the idea that such enzymes may be used in specific high-risk populations to control the reservoir of pathogenic bacteria and thus control disease. A perfect example is the prevention of secondary infections after influenza. Recent studies reveal that 50–90% of deaths resulting from influenza are due to a secondary infection caused predominantly by *S. pneumoniae*, *S. aureus*, and *S. pyogenes* in that order (Brundage and Shanks 2007; Morens et al. 2009). Reducing the carriage of these mucosal pathogens during flu season would have a significant impact on disease.

Similar to other proteins delivered intravenously to animals and humans, lysins have a short half-life ($T^{½} = 15–20$ min) (Loeffler et al. 2003). However, the action of lysins for bacteria is so rapid that this may be sufficient time to observe a therapeutic effect (Jado et al. 2003; Loeffler et al. 2003) (Fig. 4). Mice intravenously infected with type 14 *S. pneumoniae* and treated 1 h later with a single bolus of 2.0 mg of Cpl-1 survived through the 48 h endpoint, whereas the median survival time of buffer-treated mice was only 25 h, and only 20% survival at 48 h. Blood and organ cultures of the euthanized surviving mice showed that only one Cpl-1-treated animal was totally free of infection at 48 h, suggesting that multiple enzyme doses or a constant infusion of enzyme would be required to eliminate the organisms completely in this application. Similar results were obtained when animals were infected and treated intraperitoneally with lysin (Jado et al. 2003; Rashel et al. 2007). Because of lysin's short half-life, it may be necessary to modify the lysins with polyethylene glycol or the Fc region of IgG, to extend its residence time in vivo to several hours (Walsh et al. 2003). In recent studies, phage lysins have also been shown to be successful in the treatment of meningitis by adding the lysin directly into the brain intrathecally (Grandgirard et al. 2008) and endocarditis by delivering

the lysin intravenously by constant IV infusion (Entenza et al. 2005). Both these applications would also benefit from modified long-acting lysins.

Important lysins with respect to infection control are those directed to *Staphylococcus aureus* (Clyne et al. 1992; O'Flaherty et al. 2005; Rashel et al. 2007; Sass and Bierbaum 2007; Sonstein et al. 1971). However, in some cases, these enzymes exhibited low activity or were difficult to produce in large quantities. In one 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 *Staphylococcus aureus* (MRSA) both in vitro and in a mouse model. In animal experiments, the authors showed 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 only if the lysin was added up to 30 min after the MRSA. Very similar results were published recently using a lysin termed LysGH15 (Gu et al. 2011). In a more recent publication, a chimera was produced linking the catalytic domain of the Twort phage lysin with the binding domain of a PhiNM3 lysin (Daniel et al. 2010). This chimera had eliminated many of the bad features of native staphylococcal phage lysins in its activity and production.

The crucial challenge for lysins would be to determine whether they are able to cure an established infection. To approach this, a mouse pneumonia model was developed in which mice were transnasally infected with pneumococci and treated with Cpl-1 by repeated intraperitoneal injections after infection was established (Witzenrath et al. 2009). From a variety of clinical measurements, as well as morphologic changes in the lungs, it was shown that at 24 h mice suffered from severe pneumonia. When treatment was initiated at 24 h and every 12 h thereafter, 100% of the mice survived otherwise fatal pneumonia and showed rapid recovery. Cpl-1 dramatically reduced pulmonary bacterial counts and prevented bacteremia. A similar result was seen in a pneumococcal bacteremia model in which mice were pretreated intravenously (IV) with 10^8 pneumococci. Mice with established bacteremia in 18 h were treated IV with a single dose of the pneumococcal-specific lysin Cpl-1. Mice beld 15 min and 120 min after treatment showed no bacterial counts in the blood (below detectible limits) (Fig. 5).

9 Bacterial Resistance to Lysins

Though several attempts have been made, thus far bacteria that are resistant to the lytic action of native lysins have not been reported. In experiments similar to those designed to reveal antibiotic resistance, lysin resistance has not been identified. For example, exposure of bacteria grown on agar plates in the presence of low concentrations of lysin did not lead to the recovery of resistant strains even after more than 40 cycles. Organisms in colonies isolated at the periphery of a clear lytic zone created by a 10 μL drop of dilute lysin on a lawn of bacteria 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 to 20 units) in liquid culture (Loeffler et al. 2001; Schuch et al. 2002). These results

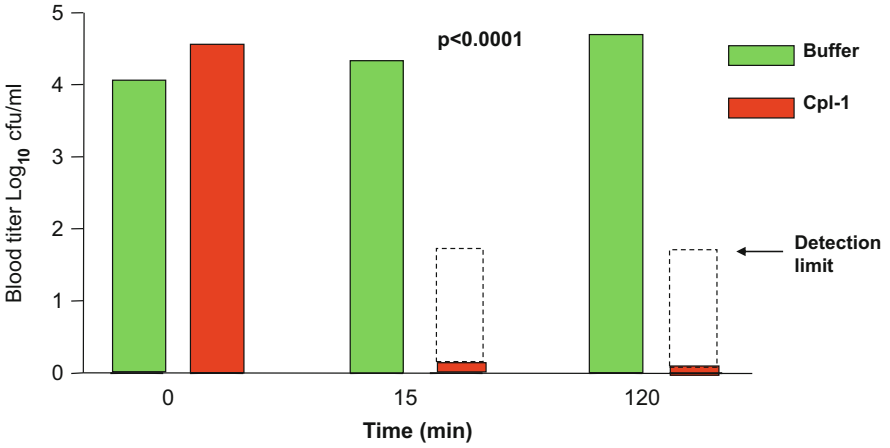

Fig. 5 Survival of bacteremic mice after treatment with Cpl-1. Mice pretreated IV with 10^8 pneumococci were bled 18 h later to determine the quantity of bacteria in the blood. Mice were then treated IV with a single 2 mg dose of Cpl-1, and groups were euthanized 15 and 120 min after treatment to determine remaining pneumococci. A single dose reduced the bacterial burden below detectible limit

may be explained perhaps by the fact that the cell wall receptor for the pneumococcal lysin Cpl-1 is choline (Garcia et al. 1983), a molecule that is essential for pneumococcal viability, and the receptor for the PlyG lysin, specific for *B. anthracis*, is the wall neutral polysaccharide, also essential for viability (Schuch et al. 2013). It is possible that lack of resistance is linked to the evolution of the binding domain, which is designed to prevent lysin spill during lysis. Since lysin spill after lysis would compromise the survival of the phage progeny, lysin binding domains evolved to bind to cell wall substrates that the bacteria could not change easily, ultimately targeting essential wall components. Because of this, resistance would be a very rare event, rarer than the frequency of antibiotic resistance.

10 Identifying and Isolating New Lysins

Gram-Positive Lysins There are a few ways in which lysins may be identified. The first and simplest is to go to the prokaryote sequence database and identify the genome sequence of the organism you are interested in killing with a lysin. Identify the phage lysin in a lysogen, synthesize the gene for that lysin and place it into an expression vector for production. If no genome sequence is available, or there are no lysogens in that species (which is rare), you will need to identify a phage from the environment for that species/organism, produce a high titer stock of the phage, shotgun clone its DNA, and identify lytic activity by overlaying the plated clones with the phage-sensitive bacterium. In this case, a lysin is identified for the organism or species that the phage infects. A more general way of isolating lysins is through

functional metagenomic analysis (Schmitz et al. 2008). This technique uses random environmental phage populations processed for metagenomic analysis. The twist here is to add an amplification step and an expression step to express and produce the products of the isolated lysin genes. This approach has the potential of identifying novel lysins with powerful biotechnological value. Another approach, which combines both the general and specific approaches mentioned above, is to exploit the lysogens in a host genome. This approach, termed multigenomics, identifies the lysin genes in the lysogens within many strains of the same species. In this case the DNA from tens to hundreds of strains of the same species are pooled and processed as in the metagenomic analysis, except here the lysins are from a single species (Schmitz et al. 2011). A related approach to multigenomics is to induce the phage from the various strains (10–50 is best) of a species with mitomycin, UV light, or some other inducing agent, pool the phage, extract the DNA, and shotgun clone to select for lysin-producing clones (Lood et al. 2015).

Gram-Negative Lysins Several lysins for gram-negative bacteria have been identified using some of the same methods used to isolate enzymes for gram-positive bacteria described above (Lai et al. 2011; Larpin et al. 2018; Lood et al. 2015).

11 Clinical Development

The need for antibiotic alternatives had been building over the last few decades and decisions need to be made regarding new therapies to combat antibiotic-resistant bacteria. In a recent review of possible antibiotic alternatives, Czaplewski et al. examined those therapies having the best probability of satisfying this need in the shortest time. They concluded, "On the basis of a combination of high clinical impact and high technical feasibility, the approaches anticipated to have the greatest potential to provide alternatives to antibiotics were phage lysins as therapeutics, vaccines as prophylactics, antibodies as prophylactics, and probiotics as treatments. . ." (Czaplewski et al. 2016).

Two companies, ContraFect and Intron Biotechnology, were the first to be in phase 1 human clinical trials in 2015 using different lysins against *S. aureus* (Czaplewski et al. 2016). In 2018, only ContraFect was actively in phase 2, testing the treatment of *S. aureus* bacteremia and endocarditis in hospitals worldwide with their lysin CF-301, (published as PlySs2 (Gilmer et al. 2013)). The results of that trial were released in early 2019. The results revealed that patients with MRSA bacteremia exhibited a 42% improvement over standard of care antibiotic treatment. Follow up studies on these patients showed that those treated with the combination of standard of care drugs plus lysin discharged from the hospital earlier and had fewer hospital requiring relapses than patients treated with standard of care drugs alone.

12 Concluding Remarks

Lysins are a new biologic to control bacterial pathogens, particularly those found on the human mucosal surface. 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, like vaccines, we should be striving to developing 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 and now gram-negative pathogens are a serious problem, such as hospitals, day care centers, nursing homes, and the environment. 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 as well as those that kill gram-negative bacteria in serum is enormous. Perhaps, someday phage lytic enzymes will be an essential component in our armamentarium against pathogenic bacteria.

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