Running Title: ENDOLYSINS AS ANTIMICROBIALS

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Daniel C. Nelson[1,2], Mathias Schmelcher[3], Lorena Rodriguez[4],Jochen Klumpp[5], David G. Pritchard[6] and Shengli Dong[6] and David M. Donovan[3]*

1 Institute for Bioscience and Biotechnology Research, University of Maryland, Rockville, MD 20850 TEL 240-314-6249, FAX 240-314-6225, nelsond@umd.edu

2 Department of Veterinary Medicine, Virginia-Maryland Regional College of Veterinary Medicine, College Park, MD 20742

3 Animal Biosciences and Biotechnology Lab, ANRI, ARS, USDA, Bldg 230, Room 104, BARC-East 10300 Baltimore Ave, Beltsville, MD 20705-2350, TEL 301-504-9150, FAX 301-504-8571, david.donovan@ars.usda.gov

4 Instituto de Productos Lácteos de Asturias (IPLA-CSIC). Apdo. 85. 33300- Villaviciosa, Asturias, Spain.

5 Institute of Food, Nutrition and Health, ETH Zurich, LFV B38.2, Schmelzbergstr. 7, 8092 Zurich, Switzerland.

6. Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, Alabama 35294

*Corresponding Author

ABSTRACT

Peptidoglycan (PG) is the major structural component of the bacterial cell wall. Bacteria have autolytic PG hydrolases that allow the cell to grow and divide. A well-studied group of PG hydrolase enzymes are the bacteriophage endolysins. Endolysins are PG degrading proteins that allow the phage to escape from the bacterial cell during the phage lytic cycle. The endolysins, when purified and exposed to PG externally, can cause "lysis from without". Numerous publications have described how this phenomenon can be used therapeutically as an effective antimicrobial against certain pathogens. Endolysins have a characteristic modular structure, often with multiple lytic and/or cell wall binding domains. They degrade the PG with glycosidase, amidase, endopeptidase, or lytic transglycosylase activities, and have been shown to be synergistic with fellow PG hydrolases or a range of other antimicrobials. Due to the co-evolution of phage and host, it is thought they are much less likely to invoke resistance. Recently, endolysin engineering has opened a range of new applications for these proteins from food safety to environmental decontamination to more effective antimicrobials that are believed refractory to resistance development. To put the phage endolysin work in a broader context, this chapter includes relevant studies of other well characterized PG hydrolase antimicrobials.

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I. Introduction

The bacterial peptidoglycan (PG) is a protective barrier as well as a structural component of the bacterial cell wall that defines its shape. Notably, the PG supports the internal turgor pressure that is essential for survival of the prokaryotic cell. PG hydrolase generically describes a wide range of lytic enzymes that act upon the bacterial PG and can be classified into several groups based on their origin. An "autolysin" is a PG hydrolase that is produced and regulated by the bacterial cell for growth, division, maintenance, and repair of the PG. In contrast, an "exolysin" is an enzyme secreted by a bacterial cell that functions to lyse the PG of a different strain or species occupying the same ecological niche. One of the most studied bacterial exolysin is lysostaphin, a PG hydrolase secreted by *Staphylococcus simulans* that cleaves the *S. aureus* PG, but does not harm the *S. simulans* PG (Schindler and Schuhardt, 1964). In addition to bacterial exolysins, eukaryotic cells can secrete their own exolysins. For example, lysozyme found in human saliva and tears is a eukaryotic exolysin that is part of the innate immune system providing protection against bacterial invasion.

PG hydrolases are also used extensively by bacteriophage (phage), for infection and/or release from a bacterial host. Particle-associated PG hydrolases can produce “lysis from without”, a term used to describe bacterial lysis in the absence of the full lytic infection cycle, as first described by Delbrück in 1940 (Delbruck, 1940). Recent work by Moak and Molineux demonstrated that PG hydrolases were associated with numerous phage particles infecting either Gram-negative or Gram-positive bacteria (Moak and Molineux, 2004). These lytic structural proteins, that are mostly tail-associated, cause localized degradation of the cell wall to enable infection of the bacterial host. Alternatively, phage encode PG hydrolases that, along with holins, are part of the lytic cassette. Holins are produced during the late stages of a phage

infection cycle to perforate the inner bacterial membrane, thus allowing the PG hydrolases that have accumulated in the cytoplasm to gain access to the PG. The result is bacterial lysis and release of progeny phage completing the infection cycle (Young, 1992). Because these PG hydrolases lyse "from within", they are referred to as "endolysins", or simply "lysins".

Significantly, exogenous addition of a phage endolysin or a bacterial exolysin to a susceptible host can be exploited to produce lysis from without due to the high osmotic pressure within the cell (~5 atmospheres for Gram-negative organisms and up to 50 atmospheres for Gram-positive organisms (Seltman and Holst, 2001)). The use of purified phage endolysins or other naturally occurring PG hydrolases as antimicrobial agents against Gram-positive pathogens is the theme of this chapter [for prior reviews, see (Callewaert et al., 2010;Fischetti, 2005;Fischetti et al., 2006;Hermoso et al., 2007;Loessner, 2005)]. Due to the presence of an outer membrane in Gram-negative bacteria, an exogenously added PG hydrolase will usually not gain access to the PG without surfactant or some other mechanism to translocate the protein across the outer membrane. Nonetheless, reports are beginning to emerge in the literature that describe fusions of Gram-negative endolysins that will lyse these pathogens from without, which will be discussed at the end of this chapter.

II. Peptidoglycan Structure

As the name implies, the peptidoglycan is a three dimensional lattice of peptide and glycan moieties. A polymer of alternating N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc) residues coupled by β(1→4) linkages comprises the “glycan” component of the PG (Fig. 1). This polymer displays little variation between bacterial species (for review see (Schleifer and Kandler, 1972)). The glycan polymer is in turn covalently linked to a short stem peptide through an amide bond between MurNAc and an L-alanine, the first amino acid of the

"peptide" component. The remainder of the stem peptide is composed of alternating L- and D-form amino acids that are fairly well conserved in Gram-negative organisms, but is variable in composition for Gram-positive organisms. For many Gram-positive organisms, the third residue of the stem peptide is L-lysine, which is crosslinked to an opposing stem peptide on a separate glycan polymer through an interpeptide bridge, the composition of which varies between species. For example, the interpeptide bridge of *S. aureus* is composed of pentaglycine (depicted in Fig. 1) whereas the interpeptide bridge of *Streptococcus pyogenes* is di-alanine. In Gram-negative organisms and some genera of Gram-positive bacteria (i.e., *Bacillus* and *Listeria*), a meso-diaminopimelic acid (mDAP) residue is present at position number three of the stem peptide instead of L-lysine. In these organisms, mDAP directly crosslinks to the terminal D-alanine of the opposite stem peptide (i.e. no interpeptide bridge). Whether an interpeptide bridge is present or not, a transpeptidation reaction joining opposing stem peptides gives rise to the three dimensional lattice that is the hallmark of the bacterial peptidoglycan. Notably, several antibiotics target the transpeptidation reaction because the crosslinking is so critical to proper formation and integrity of the cell wall and survival of the organism.

III. Endolysin Activities and Structure

A. Enzymatic activities

Due to the moderately conserved overall structure of the PG, there are limited types of covalent bonds that are available for cleavage by endolysins and other PG hydrolases (Fig. 1). In general, there are four mechanistic classes associated with PG hydrolases: glycosidase, endopeptidase, a specific amidohydrolase, and lytic transglycosylase. One type of glycosidase, known as an N-acetylglucosaminidase, cleaves the glycan component of the PG on the reducing side of GlcNAc (Fig. 1A). This type of activity is frequently found in autolysins, such as

AltA from *Enterococcus faecalis* (Mesnage et al., 2008) or AcmA, AcmB, AcmC, and AcmD from *Lactococcus lactis* (Steen et al., 2007). However, with the exception of the streptococcal LambdaSa2 endolysin (Pritchard et al., 2007), this activity has not been associated with phage endolysins. A second type of glycosidic activity is an N-acetylmuramidase, which cleaves the glycan component of the PG on the reducing side of MurNAc (Fig. 1B). This activity is commonly referred to as a "muramidase" or "lysozyme" and is frequently found in autolysins, exolysins, and phage endolysins, including the pneumococcal Cpl-1 endolysin (Garcia et al., 1987) and the streptococcal B30 endolysin (Pritchard et al., 2004).

The second class of PG hydrolases is an N-acetylmuramoyl-L-alanine amidase, a specific amidohydrolase that cleaves a critical amide bond between the glycan moiety (MurNAc) and the peptide moiety (L-alanine) of the PG (Fig. 1C) This activity is more often associated with bacteriophage endolysins than autolysins or exolysins. The reasons for this are not clear. However, because hydrolysis of this bond separates the glycan polymer from the stem peptide, such activity is speculated to be more destabilizing to the PG than hydrolysis of other bonds and may be evolutionarily favored by bacteriophage that require rapid lysis of host cells for dissemination of progeny phage. This activity has been demonstrated for the amidase domain of the staphylococcal phage Φ11 endolysin (Navarre et al., 1999), the phage K endolysin, LysK (Becker et al., 2009a;Donovan et al., 2009), and the *Listeria* phage endolysins Ply511 (Loessner et al., 1995b) and PlyPSA (Korndorfer et al., 2006).

The third class of PG hydrolases is that of an endopeptidase (i.e. protease), which cleaves peptide bonds between two amino acids. This cleavage may occur in the stem peptide, such as the listerial Ply500 and Ply118 L-alanyl-D-glutamate endolysins (Loessner et al., 1995b), or in the interpeptide bridge, such as the staphylococcal Φ11 D-alanyl-glycyl endolysin (Navarre et al., 1999) or the lysostaphin exolysin (Fig. 1D-G).

The fourth and final class of PG lytic enzymes is the lytic transglycosylase. By definition, these enzymes are not true "hydrolases" because they do not require water to catalyze PG cleavage. They are very similar to muramidases in that they cleave the β(1→4) linkages between N-acetylmuramyl and N-acetylglucosaminyl residues of the PG (Fig. 1B), but they form a 1,6 anhydromuramyl residue during glycosidic cleavage and thus belong to a different mechanistic class than the lysozymes (Holtje and Tomasz, 1975). The [T]aylor and endolysin Gorazdowska, 1974) and the gp144 endolysin from the ΦKZ bacteriophage (Paradis-Bleau et al., 2007) were both biochemically confirmed to be lytic transglycosylases.

B. Biochemical determination of endolysin specificity

Numerous studies have investigated the specificity of endolysins by assaying the cleavage sites on purified PG (Dhalluin et al., 2005;Fukushima et al., 2007;Fukushima et al., 2008;Loessner et al., 1998;Navarre et al., 1999;Pritchard et al., 2004). Classic biochemical methods, such as the Park-Johnson method, can be used to measure an increase of reducing sugar moieties as an indication of glycosidase activity by reduction of ferricyanide to ferrocyanide (Park and Johnson, 1949;Spiro, 1966). A variation of the method using sodium borohydride to reduce digested cell wall samples (Ward, 1973) has also been used frequently (Deutsch et al., 2004;Dhalluin et al., 2005;Scheurwater and Clarke, 2008;Vasala et al., 1995).

Endopeptidase or L-alanine amidase activities can be observed by an increase of free amine groups as measured by a trinitrophenylation reaction originally described by Satake (Satake et al., 1960) and modified by Mokrasch (Mokrasch, 1967). N-terminal sequencing of digestion products (i.e., Edman degradation) can also reveal cleavage sites of a PG hydrolase possessing an endopeptidase activity (Navarre et al., 1999;Pritchard et al., 2004). Alternatively, digestions products can be labeled with FDNB (1-Fluoro-2,4-dinitrobenzene) followed by HCl

hydrolysis and Reverse Phase-HPLC (Fukushima et al., 2007). HPLC peaks can be analyzed by MS and resulting fragment ions by MS-MS analysis (Fig. 2) (Becker et al., 2009a;Fukushima et al., 2008;Navarre et al., 1999)). Many of the techniques described above were used in an elegant series of experiments that showed the streptococcal phage B30 endolysin contains both a glycosidase and an endopeptidase activity within the same protein (Baker et al., 2006;Pritchard et al., 2004).

C. Confusion over historical endolysin nomenclature

The assignment of nomenclature to endolysins has been less than ideal. Decades ago, endolysins were simply referred to as “lysozymes”, a generic term often applied to PG hydrolases despite a lack of biochemical evidence characterizing their enzymatic activity. Unfortunately, many of these older designations persist to this day. The endolysin of the T7 bacteriophage continues to be called the “T7 lysozyme” in the literature despite experimental evidence dating back to 1973 showing that it is actually an N-acetylmuramoyl-L-alanine amidase rather than an N-acetylmuramidase (i.e. lysozyme) (Inouye et al., 1973). Likewise, the λ endolysin was shown to be a lytic transglycosylase 35 years ago, but the “lysozyme” moniker continues in the current literature. Another challenge is the generic classification of many endolysins simply as “amidases”, which is ubiquitously used to describe both N-acetylmuramoyl-L-alanine amidases and endopeptidases, the latter being exclusive to hydrolysis of an amide bond between two amino acids. To further complicate this issue, a protein family called CHAP (cysteine, histidine-dependent amidohydrolase/peptidase) has emerged as a common domain found in bacteriophage endolysins (Bateman and Rawlings, 2003). Experimental evidence shows the CHAP domain of the group B streptococcal B30 lysin is a D-alanyl-L-alanyl endopeptidase (Pritchard et al., 2004) whereas the CHAP domain of the group A streptococcal PlyC lysin is an N-acetylmuramoyl-L-alanine amidase (Fischetti et al.,

1972;Nelson et al., 2006). Finally, many endolysin catalytic domains are alleged to possess a particular activity based exclusively on limited homology to another endolysin domain with a putative function. When actual experiments are conducted to determine cleavage specificities, the results are often contrary to the function assigned by bioinformatic analysis. For example, in silico analysis suggests the streptococcal endolysins λSa1 and λSa2 contain N-acetylmuramoyl-L-alanine amidase activities. However, utilizing electrospray ionization mass spectrometry, Pritchard et. al., not only showed an absence of N-acetylmuramoyl-L-alanine amidase activity, but provided evidence that these enzymes function as D-glutaminyl-L-lysine endopeptidases (Pritchard et al., 2007). Clearly, more rigorous biochemical characterization of bacteriophage endolysins will help to better define and predict the catalytic classes of these enzymes.

D. Endolysin modular structure

D.1. Gram-negative endolysin structure

The Gram-negative PG, which lies subjacent to the outer membrane in the periplasmic space, is relatively thin and undecorated by surface proteins or carbohydrates. Consequently, most lysins from phage that infect Gram-negative hosts are single domain globular proteins that are typically comprised of only a single catalytic domain and have a mass of 15 to 20 kDa. However, two Gram-negative phage endolysins (*Pseudomonas* phage endolysins KZ144 and EL188) were recently shown to harbor both a lytic domain and an N-terminal cell wall binding domain (CBD) (Briers et al., 2007). The first 83 amino acids of KZ144 have been shown to be sufficient for high affinity binding to *Pseudomonas aeruginosa* cell walls (Briers et al., 2009). Moreover, this domain was shown to bind to Gram-negative PG from all species on which it was tested (after chemical treatments to remove the outer membrane) (Briers et al., 2007).

D.2. Gram-positive endolysin structure

In contrast to the Gram-negative bacteria, Gram-positive organisms contain no protective outer membrane, but rather have a much thicker (up to 40 layers) PG layer that is highly crosslinked and decorated with surface carbohydrates and proteins. Endolysins from Gram-positive infecting bacteriophage typically utilize a modular design (Diaz, et al.,1990), having one or more catalytic domains and a CBD that recognizes epitopes on the surface of susceptible organisms, often giving rise to strain- or near-species-specific binding (Schmelcher et al., 2010). Typically, a flexible interdomain linker sequence connects the catalytic domain(s) to the CBD (Korndorfer et al., 2006).

Nearly all Gram-positive endolysins and autolysins are the products of single genes, though group I introns are often found within these genes and have been reported for *Streptococcus* (Foley et al., 2000) and *Staphylococcus* (Becker et al., 2009b;Kasparek et al., 2007;O'Flaherty et al., 2004). The gene encoding the streptococcal C1 phage endolysin, PlyC, was originally believed to contain an intron (Nelson et al., 2003), but was later shown to be synthesized from two genes. This enzyme is composed of a gene product, PlyCA, which contains the catalytic domain and eight identical copies of a second gene product, PlyCB, which harbors the CBD (Nelson et al., 2006). To date, no other multimeric lysin has been identified and the implications for a multi-gene, heterononomer are not abundantly clear. Nonetheless, nanogram quantities of PlyC can achieve ~7 log killing of streptococcal cells within seconds, making PlyC several orders of magnitude more active than any other PG hydrolase ever described (Nelson et al., 2001).

The three-dimensional crystal structure of known endolysin lytic domains was reviewed recently (Hermoso et al., 2007). A very complete discussion of the PG hydrolase endopeptidase

activities and their active site structure was also recently presented by Bochtler and colleagues (Firczuk and Bochtler, 2007). Interdomain linker sequences between the catalytic and CBD domains can vary in size and can impart an inherent flexibility to these proteins making crystallography of full-length endolysins challenging. Many attempts have yielded only the structures of individual catalytic domains or isolated CBDs (Korndorfer et al., 2008;Low et al., 2005;Porter et al., 2007;Silva-Martin et al., 2010). Only a few full-length structures have become available, including PlyPSA, a listerial N-acetylmuramoyl-L-alanine amidase (Korndorfer et al., 2006), and Cpl-1, a pneumococcal N-acetylmuramidase (Hermoso et al., 2003). Remarkably, both structures reveal extreme compartmentalization displayed by the individual domains (Bustamante et al., 2010;Monterroso et al., 2008).

D.3. Domain conservation of Gram-positive endolysins

Alignment of conserved PG hydrolase domain sequences is available in public data sets (e.g. Pfam; http://pfam.jouy.inra.fr/). Such comparisons have identified numerous conserved domains shared across many genera for both binding to the bacterial surface (CBDs) and catalysis of the PG (lytic domains). Through a limited number of site-directed mutagenic studies, invariant amino acid residues conserved in domain sequences have been identified. Primarily histidine residues have been identified, that when mutated, can destroy the hydrolytic activity of the M23 endopeptidase domain (Fujiwara et al., 2005) or the cysteine, histidine-dependent amidohydrolases/peptidases (CHAP) domain (Bateman and Rawlings, 2003;Huard et al., 2003;Nelson et al., 2006;Pritchard et al., 2004;Rigden et al., 2003).

Using public data sets and Pubmed, we have attempted to compile the known PG hydrolase sequences for each of three genera *Enterococcus*, *Staphylococcus*, and *Streptococcus*. These protein structures are collated in Figs. 3, 4 and 5. This summary sheds light on the degree of

domain conservation and the range of lytic protein domain organization within and between these closely related genera. Within each genus, the endolysins have been collated into groups based on protein architecture and sequence homology. The group members are listed in Tables 1, 2 and 3. Each group has mostly > 90% within group identity at the amino acid residue level, and between group identities is mostly less than 50%. There are also stand-alone lysins with no apparent homologues yet reported. There has not been an attempt to assign a species to each of the endolysins within a genus, due to the high frequency of mobile genetic elements and lateral gene transfer that is known to exist within each (Lindsay, 2008;Palmer et al., 2010;Rossolini et al., 2010). Each of the domains listed in Figs. 3, 4 and 5 can be found in public data sets describing conserved domains (PFAM : http://pfam.sanger.ac.uk/ or NCBI conserved domain database (CDD): http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml).

D.4. Endolysins with multiple catalytic domains

Although it is well established that single domain endolysins can lyse the target pathogen (Sanz et al., 1996), there are numerous endolysins that harbor two short lytic domains (~100-200 amino acids), each encoding a different catalytic activity. A few examples of dual domain endolysins for which the cut sites are known include: 1) the staphylococcal Φ11 endolysin has both N-acetylmuramoyl-L-alanine amidase and D-alanyl-glycyl endopeptidase catalytic activities (Navarre et al., 1999), 2) the group B streptococcal lysin B30 was shown to have both N-acetylmuramidase and a D-alanyl-L-alanyl endopeptidase catalytic activity on purified PG (Pritchard et al., 2004), 3) the streptococcal λSa2 phage endolysin has an N-terminal D-glutaminyl-L-lysine endopeptidase activity and an N-acetylglucosaminidase C-terminal domain (Pritchard et al., 2007), and 4) LysK is the staphylolytic phage K endolysin featuring a CHAP endopeptidase and an amidase domain but shares less than 50% amino acid sequence identity

with the Φ11 endolysin despite cleaving identical bonds on purified staphylococcal PG (Becker et al., 2009a).

The presence of two catalytic domains does not necessarily indicate that both are equally active when lysing from without. The streptococcal λSa2 phage endolysin D-glutaminyl-L-lysine endopeptidase activity domain was shown via deletion analysis to be responsible for almost all of the hydrolytic activity of this enzyme whereas its N-acetylglucosaminidase domain was found to be almost devoid of activity (Donovan and Foster-Frey, 2008). The same dominant domain phenomena was demonstrated with both deletion and site-directed mutational analysis for the streptococcal B30 phage endolysin [99% identical to PlyGBS (Cheng and Fischetti, 2007)]. The N-terminal D-alanyl-L-alanyl endopeptidase domain is responsible for virtually all of the in vitro streptolytic activity and the glycosidase domain is silent in these assays (Donovan et al., 2006b) despite both domains showing catalytic activity on purified PG (Pritchard et al., 2004). There is no current explanation for this recurrent pattern of a highly conserved lytic domain that is seemingly inactive (when applied externally) in these unrelated streptococcal proteins (λSa2 vs. B30). These two proteins share little in the way of domain architecture (lytic-CBD-CBD-lytic vs. lytic-lytic-CBD), there are virtually no conserved sequences between them, and each utilizes an unrelated CBD (Cpl-7-like vs. SH3b).

This pattern is not limited to the streptococcal lysins. Interestingly, inactive lytic domains are also observed in staphylolytic endolysins. The staphylolytic Φ11 endolysin was shown via deletion analysis to have a very active N-terminal D-alanyl-glycyl endopeptidase domain (Donovan et al., 2006c;Sass and Bierbaum, 2007) and a nearly silent N-acetylmuramoyl-L-alanine amidase domain (Sass and Bierbaum, 2007). The staphylococcal phage endolysin LysK shares a high degree of domain architecture with the Φ11 endolysin and shows the same pattern of a highly active N-terminal CHAP endopeptidase domain (Becker et al., 2009a;Horgan

et al., 2009) and a nearly silent second lytic (amidase) domain. This pattern also shows up in numerous (but not all) SH3b containing staphylococcal endolysins (DMD unpublished data). The fact that this pattern is occurring in seemingly unrelated proteins and in more than one genera begs the question of why would this be evolutionarily conserved. A discussion of potential explanations has been presented previously (Donovan and Foster-Frey, 2008) and thus will not be repeated here, but the most likely explanation lies in the potential (unidentified) differences between lysis from without (where these nearly silent domains have been identified) vs. lysis from within. Needed are a series of experiments that test the effect of a mutant endolysin gene, with either the active or silent domain ablated, in a wild-type phage lytic cycle.

E. Measuring endolysin activity

The catabolic activity of PG hydrolases has been studied and quantified for many years. The earliest assays did not focus on antimicrobial activity but rather used PG hydrolase enzymes to degrade PG in order to elicit PG structure (Schleifer and Kandler, 1972;Weidel and Pelzer, 1964). These early studies laid the ground work for identification of the enzymes as antimicrobials. It should be noted that although multiple assays have been used to quantify the PG hydrolase activity, there can be quantitative discrepancies from assay to assay (Kusuma and Kokai-Kun, 2005). Similarly, measuring PG hydrolase enzymatic activity is not the same as measuring PG hydrolase antimicrobial activity (which by definition must assay live cells). Nonetheless, below is a list of both qualitative and quantitative assays that have been employed in the study of PG hydrolases.

Turbidity reduction assays: A decrease in light scattering (i.e., turbidity reduction) of a suspension of live cells, non-viable cells (heat killed or autoclaved), or cell wall preparation/extract can be used in a spectrophotometer to assay the activity of PG hydrolases.

The reduction in optical density over time (minutes or hours) can be used to calculate a rate of hydrolysis (Fig. 6). Results are compared to a “no-enzyme added, buffer only control” preparation treated identically for the same period of time. In this manner, a specific activity of the enzyme preparation can be reported as ΔOD/time/μg lysin protein. Critical to the interpretation of these assays include considerations for whether or not: 1) the assay is performed in the linear range of enzyme activity with excess substrate always present, 2) the maintenance of a homogeneous substrate solution (to avoid the substrate settling out of solution), and 3) the requirement for an identically treated no-enzyme control sample, the OD of which must be subtracted from the experimental sample result. There are published results using the spectrophotometric turbidity reduction assays to quantify enzyme activity (Filatova et al., 2010) and even determine kinetic constants (Mitchell et al., 2010). However, some caution should be used when interpreting the results because a loss of optical density is not always directly equated with antimicrobial activity (Fig. 6). Furthermore, variation in the assay between laboratories and arbitrary unit definitions often makes comparison of lytic activities difficult. Activities of phage-encoded and bacterial PG hydrolases reportedly range from 10^2 to 10^8 “units” per mg protein (Fukushima et al., 2007;Loeffler et al., 2003;Loessner et al., 1995a;Nelson et al., 2001;Vasala et al., 1995;Yoong et al., 2006).

Zymogram assay: Zymograms are a simple way to follow PG hydrolase activity during purification. Briefly, endolysin preparations are electrophoresed in duplicate SDS-PAGE gels. The gels are prepared either with or without the target cells or extracted PG embedded in the gel during polymerization. Following electrophoresis, the gel is soaked for 1 hour in a buffer compatible with the lytic enzyme to remove the SDS. The appearance of a cleared region in the opaque gel indicates that the cells embedded in the gel were lysed at that location, most likely due to a lytic protein/agent in the gel. This too is not an antimicrobial assay per se as the bacterial cells are often heat-treated before mixing them with the gel matrix, and are obviously

SDS-treated. Nonetheless, a zymogram is particularly useful for identifying putative PG hydrolases and offers a higher sensitivity level than the turbidity reduction assays.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC): MIC and MBC are classical assays for quantifying the antimicrobial activity of a variety of drugs. The protocols are described in detail in bacteriological manuals (Jones et al., 1985). Briefly, a 2X dilution series (100, 50, 25 µg, etc.) of the compound to be assayed (i.e. antibiotic or PG hydrolase) is established in a defined volume (usually in a 96 well plate) of growth media to which a constant number of colony forming units (CFUs) is added (i.e., 1×10^5) and incubated overnight at 37ºC. After 20 hours, the wells are examined for growth or no growth (turbid or clear) (Becker et al., 2009a). The lowest concentration of the compound that can inhibit overnight growth is the MIC (usually reported in µg/ml). For MBC, an aliquot of the wells with no apparent growth (clear to the eye) is plated onto agar growth media, and the lowest concentration of the compound that results in no CFUs (no viable cells) is the MBC (µg/ml). All PG hydrolase enzymes are not amenable to the MIC assay for reasons unknown. For these enzymes, cleared wells are never obtained, despite highly active PG hydrolase activity in multiple other PG hydrolase assays (DMD, unpublished data).

Plate Lysis (spot on lawn): A log growth-phase culture of the target bacteria is plated onto media agar plates (e.g. 0.6 ml of culture per 100 mm plate) and allowed to air dry (~15 min.) at room temp. 10 µl aliquots of known concentration(s) of the PG hydrolase are spotted onto the lawn and allowed to air dry (~10 min.) at room temp. The plates are incubated at optimal growth temperature and the plates assayed after overnight growth. A cleared spot on an opaque lawn indicates lytic antimicrobial activity of the PG hydrolase. Relative activity levels can be obtained by spotting a dilution series on the plate.

The disk diffusion assay is a variation of the plate assay, but opposed to spotting a known concentration directly onto a recently plated lawn of bacteria, a disk of sterile filter paper with a known concentration of PG hydrolase embedded in the disk is placed on the surface of the lawn and a ring of growth inhibition or lysis is observed after overnight growth. This method is not only dependent on a lytic agent, but simultaneously requires that the compound does not stick to the filter and can diffuse through the agar growth media.

Soft agar overlay assay: For screening of expression libraries for clones producing PG hydrolases, a soft agar overlay assay can be performed (Loessner et al., 1995b;Schuch et al., 2009). Replica plates containing an inducer of protein expression (e.g., IPTG) are created from original agar plates containing transformant colonies. The replica plates are incubated at 37°C for up to 6 h to allow protein production. Then, the colonies are exposed to saturated chloroform vapor for ~5 min in order to disintegrate the cytoplasmic membrane and externalize the expressed proteins, and immediately overlaid with soft agar (0.4% agar in water or buffer) containing bacterial substrate cells at high concentration. After incubation at room temperature (30 min to 18 h), lytic phenotypes can be identified by clear halos in the turbid soft agar layer. Subsequently, positive clones can be picked from original plates for plasmid isolation and genetic characterization.

Interestingly, although each of these assays can quantify the lytic activity of PG hydrolases, when a comparison of four different assays (i.e. turbidity, disk diffusion, MIC, and MBC) was utilized to quantify the antimicrobial activity of lysostaphin, the results were not always directly comparable between assays (Kusuma and Kokai-Kun, 2005). A similar result indicating qualitative but not quantitative agreement between assays was demonstrated with zymogram, turbidity reduction, MIC, and plate lysis assays using constructs of LysK, the staphylococcal

phage K endolysin (Becker et al., 2009a). A reasonable explanation for this quandary was proposed by Kusuma et al. (Kusuma and Kokai-Kun, 2005) acknowledging that bacteria express different surface factors in liquid media than on solid media (culture media can effect capsular polysaccharide production in *S. aureus*). They also suggest that the MIC assay may not be the most appropriate assay for a rapidly acting lytic enzyme, since the MIC assay measures growth inhibition while PG hydrolases probably kill the initial inocula rapidly.

F. Cell wall binding domains on Gram-positive endolysins

There are numerous domains that have been assigned CBD status (see Figs. 3, 4 and 5). Very few of these have been demonstrated unequivocally to be true CBDs. However, their ability to confer altered species-/cell wall- specificity is highly suggestive and thus CBD status has been assigned. One of the first PG hydrolase binding domains identified was the Cpl-7 choline binding domain of the pneumococcal amidase autolysin, which requires choline or ethanolamine to achieve full activation (Garcia et al., 1990). Significantly, choline moieties are distinctive of the pneumococcal cell wall. When the binding domain of a pneumococcal autolysin (amidase) was exchanged with the CBD of the phage lysozyme Cpl-7, a dependence on choline binding for enzyme activation was observed (Diaz et al., 1990;Diaz et al., 1991). Similar Cpl-7-like CBDs have been found in a Group B streptococcal λSa2 phage endolysin (Pritchard et al., 2007) that appear to be essential for lytic activity (Donovan and Foster-Frey, 2008).

Another of the most well studied PG hydrolase CBDs is that of the M23 glycyl-glycine endopeptidase, lysostaphin, and it's homologue ALE-1 that is 80% identical in both the lytic and CBDs. The lysostaphin bacterial src homology 3 (SH3b) CBD binds to the pentaglycine interpeptide bridge of the *S. aureus* PG (Grundling and Schneewind, 2006). The regions and exact amino acid residues involved in this binding have been identified in the C-terminal domain

via site directed mutagenesis of ALE-1 (Lu et al., 2006). A recent study reports that both lysozyme and lysostaphin are more active when the C-terminus of the Target of RNAIII activating protein (TRAP) is present in the staphylococcal cell wall. Binding studies indicate that the binding of these two lytic enzymes to the staphylococcal cell surface is favored by the TRAP protein C-terminus (Yang et al., 2008). Additional (SH3b) domains are found on many phage endolysins and appear to bind to the cell wall in an as yet undetermined manner.

For some species, the CBD recognition of an epitope is analogous to recognition of a cell surface receptor by a phage tail fiber. In fact, there is some evidence that these two disparate types of proteins have evolved to target identical epitopes. For example, the γ-phage of *Bacillus anthracis* forms plaques on all tested *B. anthracis* strains as well as *B. cereus* 4342, which is considered a *B. anthracis* transition state strain, but not other *B. cereus* strains (Schuch et al., 2002). Significantly, the lytic range of γ-phage endolysin, PlyG, mirrors the host range of the phage. In a similar fashion to pneumococcal phage tail fibers (Lopez et al., 1982), pneumococcal lysin CBDs are known to bind choline in the pneumococcal cell wall (Hermoso et al., 2003;Lopez et al., 1982;Lopez et al., 1997). Some CBDs of *Listeria* phage endolysins are in fact not just species-specific, but through binding to presumably teichoic acid moieties achieve serovar or even strain specificity (Kretzer et al., 2007;Loessner et al., 2002;Schmelcher et al., 2010). However, these highly specific endolysins are exceptions rather than the rule. In most cases, the specificity of the phage is more restrictive than its encoded endolysin. The C1 bacteriophage only forms plaques on group C streptococci, yet its endolysin, PlyC, efficiently lyses groups A, C, and E streptococci (Krause, 1957), as well as *Streptococcus uberis* (DCN unpublished observation). An extreme example would be PlyV12, an endolysin derived from the enterococcal phage φ1. This enzyme not only lyses *E. faecalis* and *E. faecium*, but it also lyses almost all streptococcal strains (groups A, B, C, E, F, G, L, and N streptococci*, S. uberis, S. gordonii, S. intermedius, and S. parasanguis*) as well as staphylococcal strains (*S. aureus*

and *S. epidermidus*) (Yoong et al., 2004). Similarly, the *Acinetobacter baumannii* phage ϕAB2 endolysin is reported to lyse both Gram-positive and Gram-negative bacteria (Lai et al., 2011).

IV. Gram-Positive Endolysins as Antimicrobials

A. In vivo activity

Phage endolysins have been studied extensively for half of a century, particularly those endolysins from the T-even phage that infect Gram-negative hosts. However, it has only been in the past ten years that scientists have begun evaluating the use of endolysins, specifically endolysins from phage that infect Gram-positive hosts, in animal infection models of human disease. Table 4 shows a complete list to date of all in vivo therapeutic trials that utilize bacteriophage-encoded endolysins, which are summarized below.

Fischetti and co-workers were the first to use a purified phage endolysin in an in vivo model (Nelson et al., 2001). It was found that oral administration of an endolysin (250 U) from the streptococcal C1 bacteriophage provided protection from colonization in mice challenged with 10^7 *Streptococcus pyogenes* (i.e. group A streptococci) (28.5% infected for endolysin treatment vs. 70.5% infected for PBS treatment). Furthermore, when 500 U of the streptococcal endolysin, named PlyC in a later publication (Nelson et al., 2006), was administered orally to 9 heavily colonized mice, no detectable streptococci were observed 2 hours post-endolysin treatment (Nelson et al., 2001). Based on these results, the authors coined the term "enzybiotic" to describe the therapeutic potential of not only the streptococcal endolysin, but all bacteriophage-derived endolysins.

PlyGBS is another phage endolysin that is active against group A streptococci as well as groups B, C, G, and L streptococci (Cheng et al., 2005). This enzyme was tested in a murine vaginal model of *Streptococcus agalactiae* (i.e. group B *Streptococcus*) colonization as a potential therapeutic for pregnant women to prevent transmission of neonatal meningitis-causing streptococci to newborns. A single vaginal dose of 10 U was shown to decrease colonization of group B streptococci by ~3 logs. Significantly, PlyGBS was found to have a pH optimum ~5.0, which is similar to the range normally found within the human vaginal tract. Moreover, this enzyme did not possess bacteriolytic activity against common vaginal microflora such as *Lactobacillus acidophilus.*

The most extensively studied endolysins in animal models are Cpl-1, an N-acetylmuramidase from the Cp-1 pneumococcal phage, and PAL, an N-acetylmuramoyl-L-alanine amidase from the Dp-1 pneumococcal phage. 100 U/ml of PAL was shown to cause ~4 log drop in viability in 30 seconds of 15 different *Streptococcus pneumoniae* serotypes representing multi-drug resistant isolates and those that contain a heavy polysaccharide capsule (Loeffler et al., 2001). In a mouse model of nasopharyngeal carriage, 1,400 U of PAL was shown to eliminate all pneumococci and 700 U was shown to significantly reduce bacterial counts, suggesting a dose response. In another study, Cpl-1 was shown to be effective in both a mucosal colonization model and in blood via a pneumococcal bacteremia model (Loeffler et al., 2003). Because the catalytic domains of PAL and Cpl-1 hydrolyze different bonds in the pneumococcal peptidoglycan, they were shown to be synergistic when used in combination in vitro (Loeffler and Fischetti, 2003) , which was later confirmed in vivo in a murine intraperitoneal infection model (Jado et al., 2003). In a study on the effectiveness of endolysins against in vivo biofilms, Cpl-1 was shown to work on established pneumococcal biofilms in a rat endocarditis model (Entenza et al., 2005). Infusion of 250 mg/kg was able to sterilize 10^5 cfu/ml pneumococci in blood within 30 minutes and reduce bacterial titers on heart valve vegetations by >4 log cfu/g in

2 hours. In an infant rat model of pneumococcal meningitis, a single intracisternal injection (20 mg/kg) of Cpl-1 resulted in a 3 log decrease of pneumococci in the cerebrospinal fluid (CSF) and an intraperitoneal injection (200 mg/kg) led to a decrease of 2 orders of magnitude in the CSF (Grandgirard et al., 2008). Finally, because pneumococci are often early colonizers to which additional pathogens and viruses adhere, Cpl-1 treatment of mice colonized with *S. pneumoniae* in an otitis media model was shown to significantly reduced co-colonization by challenge with influenza virus (McCullers et al., 2007).

Several phage endolysins have also been used against vegetative cells and germinating spores of *Bacillus* species. 50 U of PlyG, an endolysin isolated from the *B. anthracis* γ phage, was shown to rescue 13 out of 19 mice in an intraperitoneal mouse model of infection and extended the life of the remaining mice several fold over controls (Schuch et al., 2002). Significantly, this enzyme displayed a favorable thermostability profile and was able to remain fully active after heating to 60°C for an hour. Moreover, the extreme lytic specificity of this enzyme toward *B. anthracis* and not other *Bacillus* species was exploited for diagnostic purposes in a luminescent-based ATP assay of *B. anthracis* cell lysis. A second *Bacillus* lysin, PlyPH, is unique in that it has a relatively high activity over a broad pH range, from pH 4.0 to 10.5. This enzyme also protected 40% of mice in an intraperitoneal *Bacillus* infection model compared to 100% death in control mice (Yoong et al., 2006). Taken together, the robust and specific properties of the *Bacillus* endolysins make them amenable to therapeutic treatment and diagnostics of *B. anthracis*.

The prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) as a primary source of nosocomial infection and community-acquired MRSA as an emerging public health threat has generated a considerable amount of interest in identifying and evaluating highly active staphylococcal endolysins. The first anti-staphylococcal endolysin investigated in vivo was MV-

L, which was cloned from the ΦMR11 bacteriophage (Rashel et al., 2007). This enzyme rapidly lysed all tested staphylococcal strains, including MRSA and vancomycin-resistant clones. In vivo, 310 U of this enzyme reduced MRSA nasal colonization ~3 logs and 500 U provided complete protection in an intraperitoneal model of staphylococcal infection when administered 30 minutes post-infection. At 60 minutes post-infection, the same amount of enzyme provided protection in 60% of mice vs. controls. Another staphylococcal endolysin, ClyS, is a chimera between the N-terminal catalytic domain of the Twort phage endolysin (Loessner, et al., 1998) and the C-terminal cell wall-binding domain of the ΦNM3 phage endolysin (Daniel et al., 2010). Like MV-L, this enzyme displayed potent bacteriolytic properties against multi-drug resistant staphylococci in vitro. In a mouse MRSA decolonization model, 2-log reductions in viability were observed 1 hour following a single treatment of 960 µg ClyS. Similarly, a single dose (1 mg) of ClyS provided protection when administered 3 hours post-staphylococcal challenge in an intraperitoneal septicemia model. Notably, ClyS showed synergy in vivo with oxacillin at doses that were not protective individually against a MRSA infection model. Most recently, 50 µg of an endolysin from the GH15 phage, LysGH15, showed 100% protection in a mouse intraperitoneal model of septicemia (Gu et al., 2011) and 925 µg of CHAPk, a truncated version of LysK, affected a 2 log drop in nasal colonization of mice 1 hour post treatment (Fenton et al., 2010).

In addition to phage-encoded endolysins, a large body of in vivo work devoted to lysostaphin, a bacterial-derived exolysin, should not be overlooked. Lysostaphin, was first identified in 1964 (Schindler and Schuhardt, 1964) and the therapeutic potential of this enzyme has been studied intensely for almost 50 years. To name but a few in vivo experiments, this enzyme has been investigated in animal models of burn infections (Cui et al., 2011), ocular infections (Dajcs et al., 2001;Dajcs et al., 2002), systemic infections (Kokai-Kun et al., 2007), keratitis models (Dajcs et al., 2000), nasal colonization (Kokai-Kun et al., 2003), and aortic valve endocarditis (Climo et al., 1998;Patron et al., 1999). In addition to human disease, *S. aureus* is the major cause of

acute bovine mastitis in milking cows. As such, lysostaphin has been evaluated for therapeutic use in mouse mammary models (Bramley and Foster, 1990) and bovine mastitis models (Oldham and Daley, 1991). Transgenic mice and cows expressing mammary lysostaphin have even been produced and studied for anti-mastitic phenotypes (Kerr et al., 2001;Wall et al., 2005).

B. Immune responses

Due to the proteinacious nature of PG hydrolases and their potential use as human and animal therapeutics, we must consider potential adverse immune responses, including the generation of antibodies, to these enzymes. It is envisioned that PG hydrolases might be applied topically, to mucous membranes (oral, nasal, or vaginal cavities), intravenous, or even intramammary in the case of bovine mastitis.

To address these questions, serum antibodies were raised to phage endolysins specific to *Bacillus anthracis*, *Streptococcus pyogenes*, or *Streptococcus* pneumonia. When high titers of these antibodies were mixed in vitro with the endolysins, killing of the target microbe was slowed, but not stopped (Fischetti, 2005;Loeffler et al., 2003). Cpl-1 is a *S. pneumoniae*-specific phage lytic enzyme. In another study, Cpl-1, a pneumococcal endolysin, was injected IV three times per week into mice for four weeks resulting in positive IgG antibodies against Cpl-1 in 5 of 6 mice. Vaccinated and naive control mice were then challenged IV with pneumococci and the mice were treated IV with 200 µg Cpl-1 after 10 hours. Bacteremic titers were reduced within 1 minute to the same level in both groups of mice (Loeffler et al., 2003). Furthermore, Western blot analysis revealed that both of the phage lytic enzymes Cpl-1 and Pal elicited antibodies 10 days after a 200-µg injection in mice, but the second injection (at 20 days) also reduced the bacteremia profile 2-3 log units, indicating that the antibodies were not neutralizing

in vivo. All mice recovered fully with no apparent adverse side effects or anaphylaxis noted (Jado et al., 2003). Taken together, these studies suggest that while antibodies can be readily raised to endolysins, they do not neutralize their hydrolytic activity in vitro or in vivo.

In recent studies performed with a catheter-induced *S. aureus* endocarditis model, lysostaphin was tolerated following administration by the systemic route with minimal adverse effects (Climo et al., 1998). Rabbits injected weekly with lysostaphin (15 mg/kg) for 9 weeks by the intravenous (IV) route produced serum antibodies to lysostaphin that resulted in an eight-fold reduction in its lytic activity, consistent with earlier work (Schaffner et al., 1967), but no adverse immune response. It is believed that high purity and the absence of Gram-negative lipopolysaccharide are essential for guaranteeing a minimal host immune response.

C. Resistance development

The near-species specificity of phage endolysins avoids many pitfalls associated with broad-range antimicrobial treatments. For example, broad-range antimicrobials lead to selection for resistant strains, not just in the target pathogen, but also in co-resident commensal bacteria exposed to the drug. The acquisition of antibiotic resistance is often accomplished by transfer of DNA sequences from a resistant strain to a susceptible strain. This transfer is not necessarily species or genus limited, and can lead to commensal bacteria that are both antibiotic resistant and that can serve as carriers of these DNA elements for propagation to neighboring bacteria. Those neighboring strains (i.e., potential pathogens) with newly acquired resistance elements can emerge as antibiotic resistant strains during future treatment episodes and be further distributed in the bacterial community. Thus, in order to reduce the spread of antibiotic resistance, it is recommended to avoid subjecting commensal bacterial communities to broad-range antibiotics.

To date, there are no reports of strains resistant to phage endolysins. Two reports have attempted to identify resistant strains (summarized in (Fischetti, 2005)). In brief, three species, *S. pneumonia, S. pyogenes and B. anthracis*, were tested with repeated exposure to sublethal doses of phage endolysins specific to each species. The surviving bacteria were then challenged with a lethal dose and there was no notable change in susceptibility. In another study, *Bacillus* species were exposed to chemical mutagens that increased the frequency of antibiotic resistance several orders of magnitude. In contrast, these organisms remained fully sensitive to PlyG, a *B. anthracis* specific endolysin (Schuch et al., 2002). A likely explanation for the lack of observed resistance in endolysins as put forth by Fischetti is that the bacterial host and phage have co-evolved, such that the phage might have evolved endolysins to target immutable bonds in order to ensure its survival and release from the host (Fischetti, 2005). Thus, resistance to the phage endolysins is expected to be a very rare event.

Despite the lack of observed resistance in the phage endolysins, there are reports of resistance to other types of PG hydrolases, specifically exolysins. Lysozyme is a human exolysin with catalytic (muramidase) and cationic antimicrobial peptide activities. It is secreted by epithelial cells, is present on mucous membranes, and in the granules of phagocytes. Degradation of the bacterial peptidoglycan by lysozyme yields peptidoglycan fragments that can elicit a strong host immune response and recruitment of immune cells. Bacterial resistance to lysozyme has been accomplished through a variety of modifications that the bacteria can incorporate into the peptidoglycan backbone [for recent reviews see (Davis and Weiser, 2011) and (Vollmer, 2008)].

Similarly, there are at least two genes that can confer resistance to the lysostaphin exolysin, which targets the bonds of the staphylococcal PG interpeptide bridge. *S. simulans* produces lysostaphin and avoids its lytic action by the product of the lysostaphin immunity factor (lif) gene

[same as endopeptidase resistance gene (epr) (DeHart et al., 1995)] that resides on a native plasmid (pACK1) (Thumm and Gotz, 1997). The lif gene product functions by inserting serine residues into the PG cross bridge, thus interfering with the ability of the glycyl-glycine endopeptidase to recognize and cleave this structure. Mutations in the *S. aureus femA* gene (factor essential for methicillin resistance) (Sugai et al., 1997) result in a change in the muropeptide interpeptide cross bridge from pentaglycine to a single glycine, rendering *S. aureus* resistant to the lytic action of lysostaphin. MRSA have been shown to mutate *femA* when exposed in vitro or in vivo to sub-inhibitory doses of lysostaphin (Climo et al., 2001). Interestingly, in one report, MRSA strains that did develop resistance to lysostaphin via the *femA* gene, showed a reduced fitness compared to their parental counter parts, were five-fold less virulent in a rodent kidney infection model, and were easily treated with β-lactam antibiotics (Kusuma et al., 2007).

Schneewind and colleagues recently identified the *lyrA* (lysostaphin resistance A) that, when mutated by a transposon insertion, reduced staphylococcal susceptibility to lysostaphin (Grundling et al., 2006). Although some structural changes in PG were noted in the *lyrA* mutant, PG purified from the *lyrA* mutant was susceptible to lysostaphin and the Φ11 endolysin, suggesting that additional unidentified alterations in the *S. aureus* cell wall envelope might mediate resistance in the *lyrA* mutant.

D. Synergy

Antimicrobial synergy has been demonstrated for multiple PG hydrolases in combination with other PG hydrolases as well as numerous other classes of antimicrobials. Synergy between two PG hydrolases was shown with LysK and lysostaphin via the checkerboard assay (Becker et al., 2008;Becker et al., 2009a). This is consistent with the two enzymes having unique cut

sites. Lysostaphin has also been shown to be synergistic in the checkerboard assay with the cationic peptide antimicrobial ranalexin (Graham and Coote, 2007) and this combination has been demonstrated to be an effective surface disinfectant (Desbois et al., 2010). Lysostaphin was also shown to be synergistic with β-lactams against oxicillin-resistant MRSA. This combination is uniquely promising in that lysostaphin resistant staphylococci are generated by modifying the pentaglycine bridge of the PG, and these cell wall altered strains are often hypersusceptible to β-lactams (Kiri et al., 2002). The pneumococcal Cpl-1 endolysin is synergistic with either penicillin or gentamicin (Djurkovic et al., 2005), and with the PaI amidase (Jado et al., 2003;Loeffler and Fischetti, 2003). The phage endolysin LysH5, which has been shown to eradicate *S. aureus* in milk (Obeso et al., 2008), is synergistic with nisin (Garcia et al., 2010). Nisin was also shown to be synergistic with lysozyme against lactic acid bacteria (Chun and Hancock, 2000).Finally, ClyS, a fusion lysin described above, has been shown to be better than mupirocin at eradicating staphylococcal skin infections (Pastagia et al., 2011) and is synergistic with oxacillin (Daniel et al., 2010).

E. Biofilms

A high level of antimicrobial resistance is achieved by many pathogens through the multi-faceted changes that accompany growth in a biofilm. Biofilms are sessile forms of bacterial colonies that attach to a mechanical or prosthetic device or a layer of mammalian cells and has an extensive extracellular matrix. NIH estimates that 80% of human bacterial infections involve biofilms (http://grants.nih.gov/grants/guide/pa-files/PA-06-537.html) (Sawhney and Berry, 2009). Bacteria in biofilms can be orders of magnitude more resistant to antibiotic treatment than their planktonic (liquid culture) counterparts (Amorena et al., 1999).

Several mechanisms are thought to contribute to the antimicrobial resistance associated with biofilms: 1) delayed or restricted penetration of antimicrobial agents through the biofilm exopolysaccharide matrix; 2) decreased metabolism and growth rate of biofilm organisms which resist killing by compounds that only attack actively growing cells; 3) increased accumulation of antimicrobial-degrading enzymes; 4) enhanced exchange rates of drug resistance genes; and 5) increased antibiotic tolerance (as opposed to resistance) through expression of stress response genes, phase variation, and biofilm specific phenotype development (Emori and Gaynes, 1993;Fux et al., 2003;Keren et al., 2004;Lewis, 2001).

Little work has been done to specifically test phage endolysins for their anti-biofilm activity. Φ11 endolysin (Sass and Bierbaum, 2007) and lysostaphin have been shown to eliminate static staphylococcal biofilms (Walencka et al., 2005;Wu et al., 2003), as has LysK (O'Flaherty et al., 2005). Lysostaphin was also shown to eliminate staphylococcal biofilms in jugular vein catheterized mice (Kokai-Kun et al., 2009). Recently, the *S. aureus* SAP-2 phage endolysin SAL-2, which is nearly identical to the phage P68 endolysin, was also reported to eliminate *S. aureus* biofilms (Son et al., 2010). Alternative strategies for eradicating biofilms are necessary, including catalytic enzymes to destroy the matrix. Bacteriophage and phage lytic enzymes are a potential new source of anti-biofilm therapy (Donlan, 2008),

F. Disinfectant use

Decontamination of environmental pathogens is another area where PG hydrolases may find a niche in the marketplace. Although most disinfectants have broad-spectrum efficacy, one can envision environments where targeted decontamination of a pathogen by a narrow-spectrum endolysin would be sufficient. For example, endolysins targeting MRSA may have utility in nursing homes, surgical suites, or athletic locker rooms; endolyins effective against *Bacillus*

anthracis may be important for decontamination of suspected exposures; those against *Listeria monocytogenes* would have applications in meat-packing or food-processing facilities; and enzymes against group A streptococci could be used to reduce bacterial loads in child care settings.

Endolysins avoid several problems that are associated with chemical disinfectants. By their enzymatic nature, endolysins do not rely on potentially toxic reactive groups utilized by chemical disinfectants. As proteins, they are inherently biodegradable and non-corrosive (i.e. a "green" disinfectant). Finally, due to the high affinity of their binding domains for the bacterial peptidoglycan and their ability to concentrate on the cell surface, endolyins may not be as susceptible to dilution factors as are chemical disinfectants.

To date, the literature is sparse with examples of PG hydrolases used for disinfecting purposes. Nonetheless, lysostaphin and the cationic peptide antimicrobial ranalexin have been shown to be synergistic at killing MRSA on solid surfaces (Graham and Coote, 2007). Similarly, the same combination was found to kill MRSA on human skin within 5 minutes using an ex vivo assay (Desbois et al., 2010). In one unique application, lysostaphin attached to nanotubes and mixed with latex paint was shown to retain anti-staphylococcal properties on painted surfaces (Pangule et al., 2010)

For endolysins, only PlyC has been tested specifically as an environmental disinfectant (Hoopes et al., 2009). PlyC lyses several streptococcal species including *S. equi*, the causative organism of equine strangles disease. This highly contagious disease of horses is transmitted through shedding of live bacteria from nasal secretions and abscess drainage onto common surfaces in a stall or barn. Chemical disinfectants can be effective against *S. equi*, but inactivation by environmental factors, damage to equipment, and toxicity are of concern. PlyC

was found to be 1,000 times more active on a per weight basis (~150,000 times more active on a molar basis) than a commercially available oxidizing disinfectant. Significantly, 1 µg of PlyC was able to sterilize 10^8 cfu/ml of *S. equi* in 30 minutes. Based on these findings, the authors performed a standard battery of tests approved by the Association of Official Analytical Chemists (AOAC), including the Use Dilution Method for Testing Disinfectants and the Germicidal Spray Products Tests. PlyC passed the Use Dilution Method, which validates disinfectant claims, and was shown to eradicate or significantly reduce the *S. equi* load on equipment of various porosities commonly found in horse stables. Finally, PlyC was shown to retain effectiveness when tested in the presence of non-ionic detergents, hard water, and organic material.

G. Food safety

The use of phage and phage products for use in food safety has been reviewed recently (Hagens and Loessner, 2010;Hermoso et al., 2007;O'Flaherty et al., 2009). ListShield™ and Ecoshield™ from Intralytix and LISTEXTM from EBI Food Safety are phage preparations designed to protect food from *Listeria monocytogenes* or *E. coli.* One regulatory distinction between phage and endolysins is that phage are considered a natural product and most endolysins are purified from a recombinant expression system, thus increasing the hurdles in the approval process.

The specific use of PG hydrolases to protect food from bacterial pathogens has also been reviewed recently (Callewaert et al., 2010;Garcia et al., 2011;Loessner, 2005;Stark et al., 2010). Despite extensive exploration in this area, at this writing, there are no approved enzybiotics (endolysins) for use in/on foods for human consumption. However, approval is anticipated

eventually, in light of the acceptance in 2006 by the US, FDA for the use of *Listeria* bacteriophage on sliced meat products.

PG hydrolases are effective antimicrobials when introduced into food stuffs via transgene expression, but the safety of consumption of transgenic food products is still a highly debated topic worldwide. Transgenic goat milk containing human lysozyme could protect from mastitis in vitro and showed benefits in animal health for goats drinking the transgenic milk (Maga et al., 2006b;Maga et al., 2006a). Similarly, pigs (Tong et al., 2010) and cattle (Yang et al., 2011) expressing lysozyme in the mammary gland have been created. Lysostaphin transgenic cattle were also protected from an intramammary *S. aureus* challenge (Wall et al., 2005). A human lysozyme expressing vector for injection into cattle mammary glands has also been created and reported to reduce mastitis symptoms within days (Sun et al., 2006).

Expression of PG hydrolases in plants might serve multiple purposes: first, as a final stage to protect food products from food pathogens or a method to protect crop production from plant pests and finally, plant systems might be a better source of the PG hydrolase in quantities needed for commercialization as opposed to fermentation-derived recombinant proteins. Potatoes can be protected from the phytopathogen *Erwinia amylovora* by the transgenic expression of the T4 lysozyme (During et al., 1993). Transgenic rice expressing human lysozyme has also been created (reviewed in (Boothe et al., 2010)) as have transgenic plants expressing a group B streptococcal endolysin, which was highly expressed in the chloroplasts (Oey et al., 2009).

Non-transgenic uses of PG hydrolases in food applications are limited. Surface application of the phiEa1h (T4 lysozyme) endolysin on pears reduced the effects of an *Erwinia* challenge (Kim et al., 2004). The staphylococcal phage endolysin LysH5 killed *S. aureus* in pasteurized milk in

vitro (Obeso et al., 2008) and was recently shown to be synergistic with nisin, a lactococcal bacteriocin that has achieved GRAS status (generally recognized as safe) (Garcia et al., 2010). A fusion of a streptococcal B30 endolysin and lysostaphin was also able to kill both streptococci and staphylococci in milk products (Donovan et al., 2006a). An endolysin from *Clostridium tyrobutyricum* (Mayer et al., 2010), which produces cheese spoilage, is also active in milk. Other clostridial endolysins that kill food pathogens have been reported (Zimmer et al., 2002;Simmons et al., 2010). Lactic acid bacteria engineered to secrete lysostaphin and a *Listeria* endolysin (Tan et al., 2008;Turner et al., 2007) or *Listeria* endolysin alone (Gaeng et al., 2000;Stentz et al., 2010) or *Clostridium* endolysin (Mayer et al., 2008) have been produced, but the ability to protect food stuffs from these pathogens has not yet been reported.

A very relevant role that endolysins play in food safety is based on the high specificity of their CBDs. These recognition domains have been used to develop rapid and sensitive identification, detection and differentiation systems (Fujinami et al., 2007;Schmelcher et al., 2010). Magnetic beads coated with recombinant CBDs enabled immobilization and recovery of more than 90% of *L. monocytogenes* cells from food samples (Kretzer et al., 2007;Walcher et al., 2010).

V. Engineering Endolysins

A. Swapping and/or combining endolysin domains

There are numerous examples in the literature of engineered PG hydrolases that range from site-directed mutant constructs used to identify essential amino acids in catalytic or CBD domains, to novel fusion constructs for the purpose of making a better antimicrobial. Some of the earliest fusions were created by the exchange of CBDs of pneumococcal autolysins and phage endolysins (Diaz et al., 1991;Garcia et al., 1990). Fusion of clostridial or lactococcal N-

acetylmuramidase catalytic domains to choline binding domains from pneumococcal endolysin CBDs resulted in choline dependence of the chimeric enzyme (Croux et al., 1993a;Croux et al., 1993b;Lopez et al., 1997). In a reverse approach, a clostridial CBD was fused C-terminally to a catalytic domain of the pneumococcal autolysin LytA, considerably increasing its activity against clostridial cell walls (Croux et al., 1993a). In another study, the catalytic domain of the lactococcal phage Tuc2009 gained activity against choline-containing pneumococcal cell walls by fusion to the CBD of LytA (Sheehan et al., 1996). The ability to swap catalytic and CBDs is not limited to choline-binding domains. The exchange of *Listeria* phage endolysin CBDs of different serovar specificity resulted in swapped lytic properties of the chimeras and enhanced lytic activity against certain strains (Schmelcher et al., 2011). In the same study, heterologous tandem CBD constructs were shown to combine the binding properties of both individual CBDs, providing them with extended recognition properties. Furthermore, a duplication of a CBD resulted in a 50-fold increase in affinity to the listerial cell wall, making this protein a useful tool for bacterial detection. Combined with an enzymatically active catalytic domain, this increased affinity resulted in enhanced lytic activity at high ionic strength. Another chimeric endolysin (P16-17) was recently constructed with the N-terminal predicted d-alanyl-glycyl endopeptidase domain and the C-terminal CBD of the *S. aureus* phage P16 endolysin and the P17 minor coat protein, respectively. This approach was also a domain-swap which greatly improved the solubility of the fusion over the parental hydrolases, allowing purification and experiments to demonstrate strong antimicrobial activity towards *S. aureus* (Manoharadas et al., 2009).

A series of intergeneric PG hydrolase fusions between the streptococcal B30 endolysin and the staphylolytic lysostaphin demonstrate activity against both pathogens (Donovan et al., 2006a). These constructs relied on the streptococcal and staphylococcal lytic domains maintaining their parental specificities, with just the lysostaphin SH3b CBD. This dual lytic specificity challenges the dogma wherein the SH3b domain was believed to be essential for endolysin specificity

(Baba and Schneewind, 1996). More recently, this theme has been expanded to include the streptococcal phage λSa2 endolysin CHAP endopeptidase domain fused to the ~92 amino acid staphylococcal SH3b CBDs from either lysostaphin or LysK. These constructs show full activity against both streptococcal and staphylococcal pathogens in numerous in vitro assays (Becker et al., 2009b), presumably due to the conserved bonds that this lytic domain recognizes and cleaves (γ-D-glutaminyl-L-lysine) in both streptococcal and staphylococcal PG. Again, the staphylococcal SH3b CBDs enhanced lytic activity on cell walls of both genera. This dual activity argues against genera- or species-specific binding of the lysostaphin SH3b domain as has been reported (Grundling and Schneewind, 2006;Lu et al., 2006).

A more recent fusion, ClyS, described above is reported to be effective at curing murine topical infections of *S. aureus* (Pastagia et al., 2011) and effective in combination with classical antibiotics at eradicating multi-drug resistant strains of *S. aureus* in a mouse model of nasal colonization (Daniel et al., 2010).

Other more trivial modifications of PG hydrolases have also been reported, such as the addition of a His-tag for ease of purification. Although such tags are considered a minor modification, rarely has the effect of such a modification been examined on lytic activity. One recent study has examined the effect of an N- or C-terminal His tag on lysostaphin with the resultant activities being 80% and 20% of the non-tagged version, respectively (Becker et al., 2011). That same publication also looked at micro deletions (6 amino acid increments) in the N-terminus of lysostaphin. Deletion of the first 3 or 6 residues has no significant effect on minimum inhibitory concentration (MIC), whereas deletion to residue 11 reduces the MIC to ~40% of wild-type with decreasing MICs for larger deletions. The lack of reproducibility of quantitative results between PG hydrolase assays for lysostaphin was first described by Kusuma et al, (Kusuma and Kokai-Kun, 2005) and that finding was confirmed recently with turbidity reduction and plate lysis

assays where N-terminal micro-deletions of lysostaphin did not show significant reduction in lytic activity until 21 residues were deleted resulting in only 17% of wild-type activity (Becker et al., 2011).

Other minimally altered constructs are those where single amino acids are purposefully altered to examine the effect on lytic activity. Pritchard and colleagues altered conserved amino acids in the streptococcal B30 endolysin CHAP and lysozyme domains, which resulted in sequential loss of activity from each domain. When analyzed on live bacteria, it was made clear that the B30 endolysin CHAP domain was the primary source of lytic activity from this dual domain endolysin when lysing 'from without' (Donovan et al., 2006b). Site-directed mutagenesis and deletion analysis of the *Bacillus anthracis* phage lysin PlyG were essential in defining the binding domain and active site residues (Kikkawa et al., 2007;Kikkawa et al., 2008), as for PlyC that was also examined in this way (Nelson et al., 2006). Similarly, site-directed mutations altering histidine codons in the staphylococcal glycyl-glycine PG hydrolase ALE-1 have been used to define essential amino acids in the M23 endopeptidase domain (Fujiwara et al., 2005). Mutations of the ALE-1 CBD when fused to GFP were used to define those amino acids essential for cell wall binding (Lu et al., 2006).

Further site-directed mutations of lysostaphin were examined when a lysostaphin transgene was expressed in the mammary gland of both mice (Kerr et al., 2001) and dairy cattle (Wall et al., 2005). Transgenic lysostaphin showed reduced activity due to N-linked glycosylation (Kerr et al., 2001). Subsequently, two Asn codons (residues 125 and 232) were modified to encode Glu in order to ablate the N-linked glycosylation. The result was a secreted functional lysostaphin, however, with a 5-10 fold reduction in lytic activity compared to wild-type lysostaphin (Kerr et al., 2001). It was recently shown by separating the two altered residues on separate constructs, that the N125Q modification alone was primarily responsible for this

reduction in activity (Becker et al., 2011). By homology to the well characterized LytM (a closely related LAS metalloprotease) (Firczuk et al., 2005), residue 125 is likely to reside in the catalytic domain of lysostaphin, and thus may alter the enzymes ability to bind the substrate. When mapped to the crystal structure of LytM (Firczuk et al., 2005) in the presence of a substrate analogue bound to a glycine rich loop in the active site cleft, mutation of the equivalent residue (LytM N303Q) added an additional carbon into the side chain in the predicted active site. It is predicted that this might crowd the substrate analog and therefore interfere with substrate binding in the active site cleft (Firczuk et al., 2005; Becker et al., 2011).

Numerous engineered truncations of PG hydrolases have been described in the literature that were created primarily for defining active residues in lytic domains. A partial list includes the Twort endolysin (Loessner et al., 1998), B30 endolysin (Donovan et al., 2006b), λSa2 endolysin (Donovan and Foster-Frey, 2008), Φ11 endolysin (Donovan et al., 2006c;Sass and Bierbaum, 2007), and the *Bacillus amyloliquifaciens* endolysin (Morita et al., 2001). Some of these efforts have yielded truncations with a greater lytic specific activity than the full length PG hydrolase e.g. the staphylococcal LysK (Horgan et al., 2009). One such hyper-active truncation construct was the result of a random mutagenesis experiment which also resulted in the incorporation of unpredicted sequences at the C-terminus of the streptococcal PlyGBS endolysin (Cheng and Fischetti, 2007). The authors suggest this enhanced activity may be potentially due to both a reduced size and the lack of full-length CBD, allowing the enzyme to move more quickly between substrate binding sites and thus lyse more cells. Other studies suggest that the presence of a CBD increases lytic activity of an endolysin, presumably by bringing the catalytic domain in proximity of its substrate (Korndorfer et al., 2006). However, duplication of a CBD, which results in a significant increase in binding affinity, was shown to reduce activity at physiological salt concentration, which again may be explained by a loss of surface mobility (Schmelcher et al., 2011).

The numerous works with fusion constructs further verify that PG hydrolases have evolved a modular design, with both lytic and CBD domains as first proposed by Diaz et al. (Diaz et al., 1990). When fused, these lytic domains can maintain their parental specificities for the PG bond cleaved, and the species of cell wall recognized. These enzymes are candidate antimicrobials for the reasons outline above, but most importantly, despite repeated attempts to identify them, no strains of host bacteria have been reported that can resist the lytic activities of their bacteriophage endolysins (Fischetti, 2005). In addition, numerous phage endolysins harbor dual lytic domains (See Figs. 3, 4 and 5). Dual domain endolysins are predicted to be more refractory to resistant strain development (Fischetti, 2005). The Donovan lab has taken this one step further and reasoned that three lytic domains might create an antimicrobial that would be even more refractory to resistance development. In theory, it is very rare that a bacterium can evade three, unique, simultaneous antimicrobial activities.

We have created several triple-lytic-domain anti-staphylococcal fusion constructs using the synergistic enzymes LysK and lysostaphin. Lysostaphin and LysK collectively harbor three unique cleavage sites that are known (described above). LysK and lysostaphin are also known to be active against multiple MRSA strains. The LysK-Lyso triple lytic domain construct described previously (Becker et al., 2009b) is highly active against both *S. aureus*, MRSA and numerous coagulase negative staphylococci (unpublished data). Most importantly, all three lytic domains are active in the fusion construct, as demonstrated by electron spray ionization mass spectrometry of PG digestion products (Donovan et al., 2009). Studies are underway to determine the efficacy of these and other triple-lytic-domain fusion endolysins in animal models of staphylococcal infection, and to test for resistant strain development both in vitro and among the staphylococci retrieved from in vivo models.

B. Fusion of endolysins to protein transduction domains

It is apparent that the high antimicrobial resistance of some persistent pathogens is due to their ability to invade and reside intracellularly within eukaryotic cells. Some examples of bacteria that utilize this niche are *Legionella pneumophila, Mycobacteria turberculosis, Listeria monocytogenes* (Vazquez-Boland et al., 2001) and S. *aureus*. There are numerous strategies that these intracellular residents have devised, including the creation of specialized vacuoles that block phagosome maturation into a phagolysosome and inhibition of phagosome acidification, to name a few (Garcia-del and Finlay, 1995). Recent works describe alternative drug treatment systems for delivery of antimicrobials to intracellular pathogens (Imbuluzqueta et al., 2010).

One recently proposed method involves fusing cell-penetrating peptides (CPPs) or protein transduction domains (PTDs) to PG hydrolases to enable these lytic enzymes access to intracellular bacteria (Borysowski and Gorski, 2010). CPPs or PTDs are usually highly positively charged regions that exist in naturally occurring proteins and are essential for the uptake of these proteins into target cells. The uptake mechanisms are likely cell type and peptide specific with some CPPs and their cargo traversing the membrane without involving pinocytosis while others require pinocytotic uptake (Duchardt et al., 2007;Joliot and Prochiantz, 2004). There are reports of non-charged peptide fragments that can also enhance transduction across the eukaryotic membrane, and some antimicrobial peptides can serve as CPPs and vice versa (Splith and Neundorf, 2011).

There are numerous reports on the use of CPPs to deliver bioactive molecules to a variety of cell types. Although no formal report exists in the literature for a PG hydrolase fused to a PTD for killing intracellular pathogens, there has been one patent application filed in 2009 wherein

lysostaphin was fused to the HIV Transactivator of transcription (TAT) protein transduction domain, Lyso-TAT (http://www.pat2pdf.org/patents/pat20110027249.pdf). In this application, the Lyso-TAT construct is reported to eradicate *S. aureus* ex vivo in cultured MAC-T mammary epithelial cells, bovine brain epithelia, human keratinocytes and murine osteoblasts.

VI. Gram-Negative Endolysins as Antimicrobials

A. Background

The use of bacteriophage-encoded endolysins, or any type of PG hydrolase, to control Gram-negative pathogens has been very limited. Their effectiveness when added exogenously is hindered by the presence of the Gram-negative outer membrane that is highly effective at excluding large molecules and not present on Gram-positive cells. The endolysin-susceptible PG layer resides between an inner and outer membrane in Gram-negative organisms, and as such, is not directly exposed to the extracellular environment. An effective strategy to allow endolysins to translocate the outer membrane is vital for their use against Gram-negative pathogens.

There are numerous studies on the use of peptides, detergents, and chelators that can be used to permeabilize the Gram-negative outer membrane in combination with PG hydrolases (Vaara, 1992). As an example, 10 mM EDTA used in combination with 50µg/ml of the *Pseudomonas* endolysin EL188 decreased viable *P. aeruginosa* cells by 3 or 4 logs in 30 minutes depending on the strain tested (Briers et al., 2011). Additionally, there have been studies in which various chemical moieties have been conjugated to PG hydrolases or hydrophobic peptides have been genetically fused to them in order to alter membrane permeability to these enzymes (Ito et al., 1997;Masschalck and Michiels, 2003). All of these strategies can be applied to bacteriophage-

derived endolysins and several specific examples are provided in the next section. However, each strategy also poses questions regarding their efficacy, practicality, and toxicity that must be determined empirically. Appreciably, agents that destabilize the Gram-negative outer membrane often destabilize eukaryotic cell membranes, both of which are similar lipid bilayers.

B. Non-enzymatic domains and recent successes

Some PG hydrolases and endolysins can kill pathogens via a mechanism completely separate from their ability to enzymatically cleave the PG. For example, heat-denatured bacteriophage T4 lysozyme was found to retain 50% of its microbicidal activity despite a complete absence of muramidase activity (During et al., 1993). The authors further identified three positively charged, amphipathic helices and show that one of them, A4, exhibits 2.5 times more killing of E. coli than intact T4 lysozyme. A4 is proposed to act by membrane disruption due to its cationic nature. This action may be similar to that of other positively charged, amphipathic helices collectively referred to as host-defense peptides (Sahl and Bierbaum, 2008).

Similar to the T4 lysozyme, several additional endolysins have been identified which contain amphipathic or highly cationic regions in addition to their catalytic domains. The preliminary studies suggest these endolysins are capable of producing lysis from without in a variety of Gram-positive and Gram-negative species. For example, LysAB2, the endolysin from the ΦAB2 *A. baumanni* phage, was found to degrade isolated cell walls of *A. baumanni* and *S. aureus* in a zymogram (Lai et al., 2011). On live, viable cells, this enzyme was shown to be antibacterial toward several Gram-negative (*A. baumanni, E. coli, Salmonella enterica*) and Gram-positive (*Streptococcus sanguis*, *S. aureus*, *Bacillus subtilis*) strains. Significantly, LysAB2 contains a C-terminal amphipathic region that was shown by deletion analysis to be necessary for the observed antibacterial activity. A second example is the lys1521 endolysin from a *Bacillus*

amyloliquefaciens phage, which possesses two cationic C-terminal regions. Using either a synthesized peptides of these regions or a catalytically inactive mutant of the endolysin, the cationic regions alone were shown to be able to permeabilize the outer membrane of *P. aeruginosa*, a Gram-negative pathogen (Muyombwe et al., 1999). The wild-type enzyme, containing an N-terminal catalytic domain and the two C-terminal cationic domains, displayed antibacterial activity against live *P. aeruginosa* (Orito et al., 2004).

These recent successes have inspired renewed interest in the use of endolysins against Gram-negative bacteria, an idea once considered a non-starter. Indeed, several new patents have been issued, which provide forward-looking insight into where the field is headed (see patents WO/2010/149792 and WO/2011/023702). It is expected that research focused on fusing endolysin catalytic domains with cationic peptides, polycationic peptides, amphipathic peptides, sushi peptides, hydrophobic peptides, defensins, and other antimicrobial peptides in the hopes of expanding endolysin-based therapy to Gram-negative pathogens will greatly expand in the coming years.

C. High-pressure treatment

In another approach, the use of high hydrostatic pressure (HHP) can dramatically increase access of phage endolysins to the Gram-negative PG. While this may not have direct human applications, it does have potential applications for decontamination and food processing. HHP has several advantages: it can be bactericidal alone (Briers et al., 2008;Hauben et al., 1996;Masschalck et al., 2000;Masschalck et al., 2001;Nakimbugwe et al., 2006), it does not use heat so it will not compromise the quality of foodstuffs, and most importantly, it is not considered to be a food additive. However, generating the required high pressures (200 to 500 MPa) can pose a cost hurdle. HHP has been used with a variety of antibacterials including nisin,

lactoferrin, and several PG (Briers et al., 2008;Hauben et al., 1996;Masschalck et al., 2000;Masschalck et al., 2001;Nakimbugwe et al., 2006).

Nakimbugwe and colleagues tested HHP in conjunction with six individual PG hydrolases, including phage endolysins from λ and T4, on ten different bacterial strains (five each of Gram-negative and -positive) (Nakimbugwe et al., 2006). Both phage endolysins were active on four out of five of the Gram-negative bacteria and *Bacillus subtilis,* though the λ-derived endolysin showed greater activity on most of the strains. In a separate study, the efficacy of hen egg white lysozyme, a PG hydrolase, and the λ lysozyme, an endolysin, were tested in conjunction with HHP on skim milk (pH 6.8) and banana juice (pH 3.8) with four Gram-negative bacteria: *E. coli O157:H7, Shigella flexneri, Yersinia enterocolitica, and Salmonella typhimurium* (Nakimbugwe et al., 2006). The λ lysozyme outperformed the PG hydrolase in a bacterial inactivation assay by almost 2 and 5 logs in skim milk and banana juice, respectively.

VII. Concluding Remarks

Multi-drug resistant super-bugs have 'raised the bar' establishing a higher set of requirements for new antimicrobials. New antimicrobial agents should ideally eradicate multi-drug resistant pathogens, including those in biofilms, and successfully prevent further resistance development. PG hydrolases and their fusions have unique properties that make them ideal candidates for this much needed new class of therapeutics. PG hydrolases usually target a narrow range of closely related pathogens, avoiding selective pressures on un-related commensal bacteria. They also target the cell surface and thus avoid the many resistance mechanisms that operate within the cell (e.g. modification of target, modification of agent, pumps to extrude the agent). PG hydrolases are effective against growing cells but can also target non-dividing or slowly growing cells e.g. biofilms, which most antibiotics cannot. The modular nature of the phage

endolysins and other PG hydrolases allow for naturally occurring and engineered lysins with two or more simultaneous lytic activities. It is expected to be a rare event that any pathogen can evade three simultaneous lytic activities. It is also worth noting that the ability to confer intracellular killing via PG hydrolase fusions to protein transduction domains is non-trivial in light of the toxic levels required for most drugs to eradicate pathogens residing intracellularly. Similarly, the PG hydrolases are synergistic with many classes of classical antimicrobials, thus potentially extending the clinical half-life of over-used antibiotics. Although there are many advantages conferred by killing a drug-resistant pathogen via a lytic enzyme that lyses from without, the reality of increased antigen release that accompanies lysis of a systemic pathogen cannot be ignored. Similarly, the inherent hurdles of production costs and antigenicity of a protein antimicrobial are still awaiting full debate in the commercialization arena. However, despite these concerns, it is clear that biofilms are the major threat in human infectious disease with NIH estimating 80% and CDC estimating 65% of human infections are in the form of biofilms. It is also clear that conventional antimicrobials are poor eradicators of biofilms, and that catalytic enzymes of some sort are going to be required to dissolve and eradicate persistent biofilms. Thus, the antigenicity of both the digestive enzyme used to treat the biofilm, and the surge of bacterial antigens released upon cell lysis or biofilm degradation are hurdles that will need to be overcome in the unavoidable assault on bacterial biofilms. We believe that PG hydrolases are an ideal candidate class of novel antimicrobials with which to address these inevitable concerns.

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Figure Legends:

Fig. 1. Structure of *S. aureus* bacterial PG and cleavage sites by PG hydrolases. (A) An N-acetylglucosaminidase hydrolyzes the glycan component of the PG on the reducing side of GlcNAc. (B) In contrast, an N-acetylmuramidase (also known as “muramidase” or “lysozyme”) hydrolyzes the glycan component of the PG on the reducing side of MurNAc. Likewise, lytic transglycosylases cleave the same bond, but form 1,6 anhydromuramyl intermediates during cleavage. (C) An N-acetylmuramoyl-L-alanine amidase cleaves a critical amide bond between the glycan moiety (MurNAc) and the peptide moiety (L-alanine) of the cell wall. This activity is sometimes referred to generically as an “amidase”. (D,E,F,G) An endopeptidase cleaves an amide bond between two amino acids. This type of activity may occur in the stem peptide of the PG, as in the case of the Listeria endolysins, Ply500 and Ply118 (D), or the streptococcal endolysin, λSa2 (E). Alternatively, an endopeptidase can cleave the interpeptide bridge as displayed by the staphylococcal endolysin Φ11 (F) or the staphylococcal bacteriocin, lysostaphin (G) Note, the structure of the Staphylococcus aureus PG is depicted for illustration purposes. Other bacterial species have interpeptide bridges composed of different amino acids or may lack an interpeptide bridge all together. In these organisms, a meso-diaminopimelic acid replaces L-Lys and directly crosslinks to the terminal D-Ala of the opposite peptide chain.

Fig. 2. Electron spray ionization mass spectrometry determination of LysK and phi80α endolysin cut sites in *S. aureus* PG. Purified *S. aureus* PG was digested with LysK and phi80α endolysin under identical conditions as described in Becker et al. (Becker et al., 2009). The digests were filtered through 5K cutoff ultrafilters and these filtrates were further processed through disposable charcoal columns (CarboPak). The bound muropeptides were eluted with 50% acetonitrile and subjected to mass spectrometry.

Fig. 3. Staphylococcal PG hydrolase structure. White boxes represent cell wall binding domains. SH3b: bacterial Src homology 3 domain (Whisstock and Lesk, 1999;Ponting et al., 1999); PGRP: Peptidoglycan recognition protein (Dziarski and Gupta, 2006). Scale bar represents the number of amino acids. * weak PFAM homology; ** not present in mature protein

Fig. 4. Streptococcal PG hydrolases. White boxes represent cell wall binding domains. ChBD: Choline binding domain (Hermoso et al., 2003); Cpl-7: (Garcia et al., 1990); SH3b: bacterial Src homology 3 domain (Whisstock and Lesk, 1999;Ponting et al., 1999). Scale bar represents the number of amino acids.

Fig. 5. Enterococcal PG hydrolases. LysM: (Bateman and Bycroft, 2000); (Joris et al., 1992); SH3b: bacterial Src homology 3 domain (Whisstock and Lesk, 1999;Ponting et al., 1999); NLP_P60: (Anantharaman and Aravind, 2003). Scale bar represents the number of amino acids.

Fig. 6. A reduction in turbidity equates to reduced bacterial viability. A. 25 µg of Φ11 endolysin (construct Φ11-194; (Donovan et al., 2006)) protein (circles) and *S. aureus* cells alone (squares) were monitored for 120 min in a turbidity reduction assay. B. Treated (Φ11-194) and non-treated (cells alone) turbidity assay samples were serially diluted and at 0, 30 and 120 min. plated onto tryptic soy agar plates. The results shown reflect the CFU/ml of the treated cells expressed as a percentage of the viable counts of the untreated control sample. Error bars = SEM.

Figure 1.

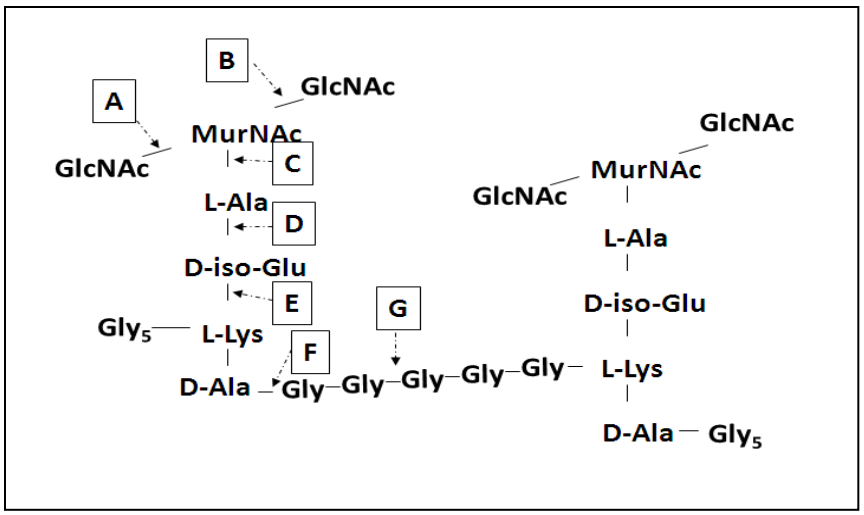


Figure 2

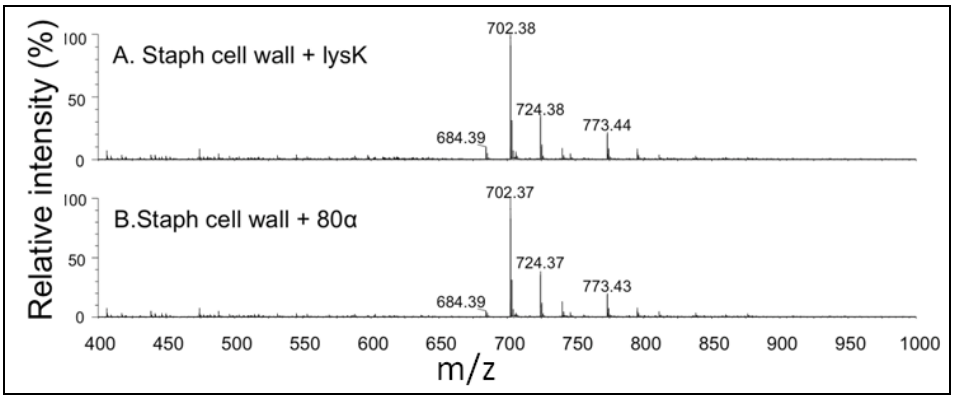
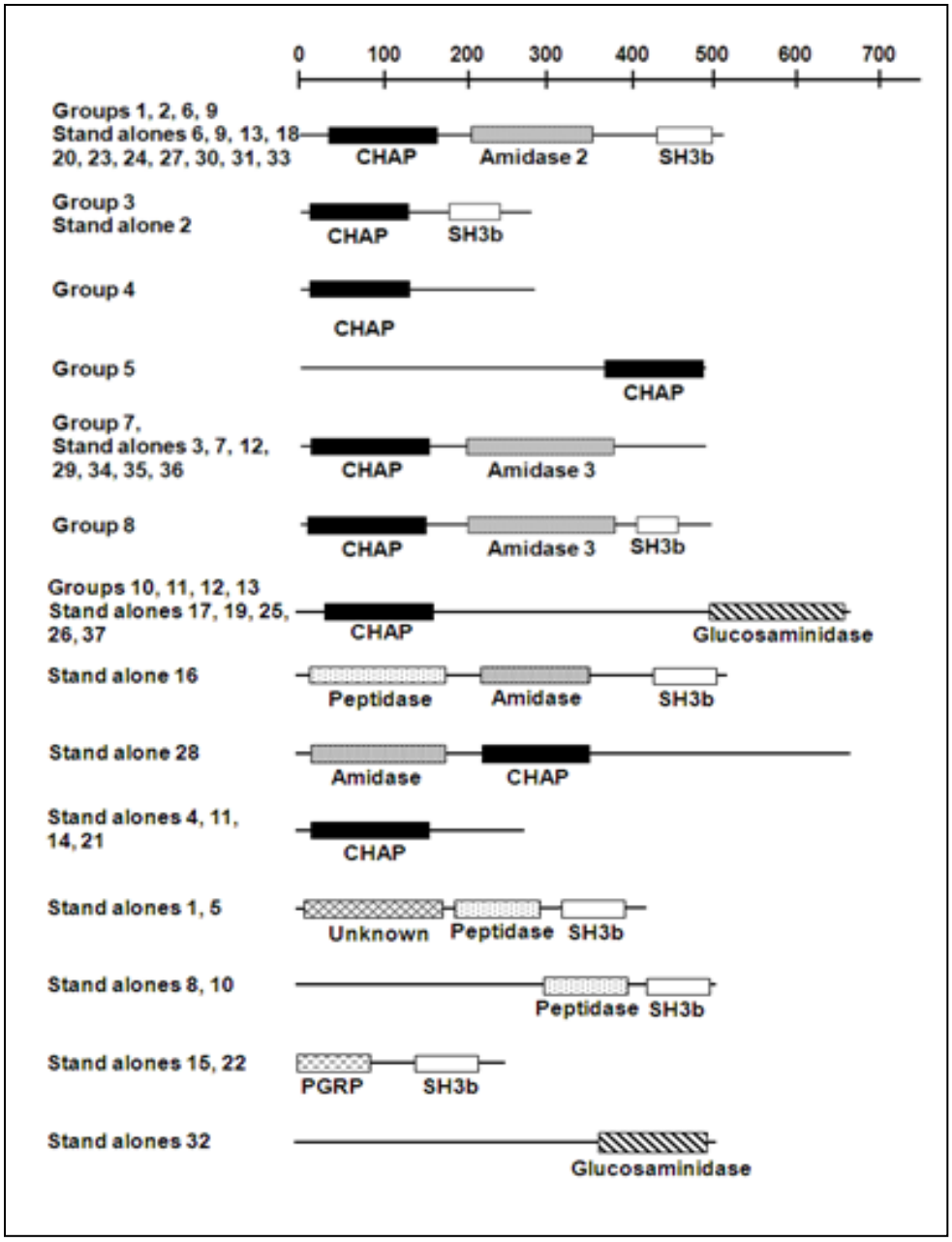


Figure 3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CHAP
Amidase 2
SH3b
Group 3
Stand alone 2
CHAP
SH3b
Group 4
CHAP
Group 5
CHAP
Group 7,
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29, 34, 35, 36
CHAP
Amidase 3
Group 8
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SH3b
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26, 37
CHAP
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Amidase
SH3b
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14, 21
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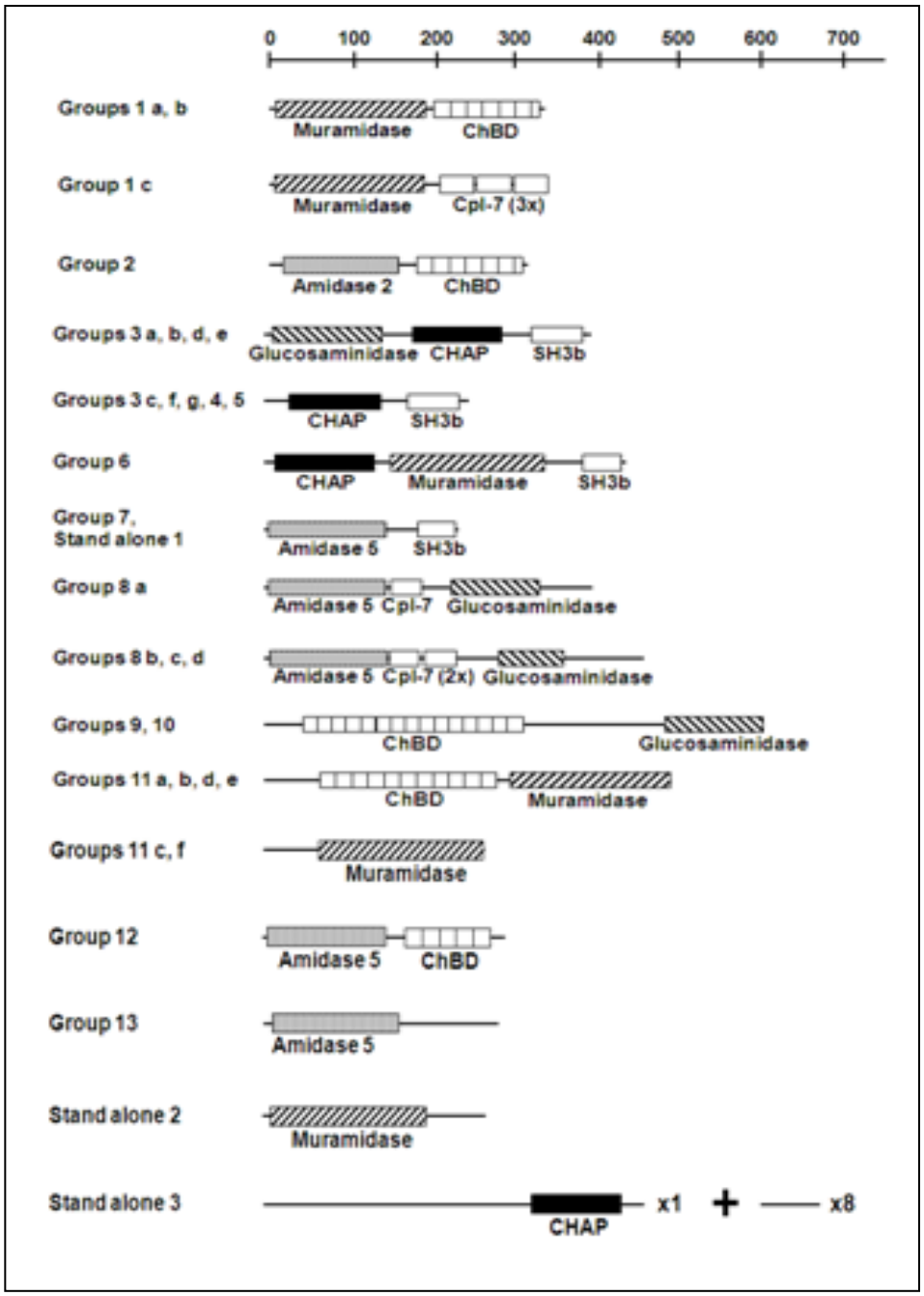
Stand alones 32
Glucosaminidase

Figure 4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ChBD
Group 1 c
Muramidase
Cpl-7 (3x)
Group 2
Amidase 2
ChBD
Groups 3 a, b, d, e
Glucosaminidase
CHAP
SH3b
Groups 3 c, f, g, 4, 5
CHAP
SH3b
Group 6
CHAP
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SH3b
Group 7,
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Amidase 5
SH3b
Group 8 a
Amidase 5
Cpl-7
Glucosaminidase
Groups 8 b, c, d
Amidase 5
Cpl-7 (2x)
Glucosaminidase
Groups 9, 10
ChBD
Glucosaminidase
Groups 11 a, b, d, e
ChBD
Muramidase
Groups 11 c, f
Muramidase
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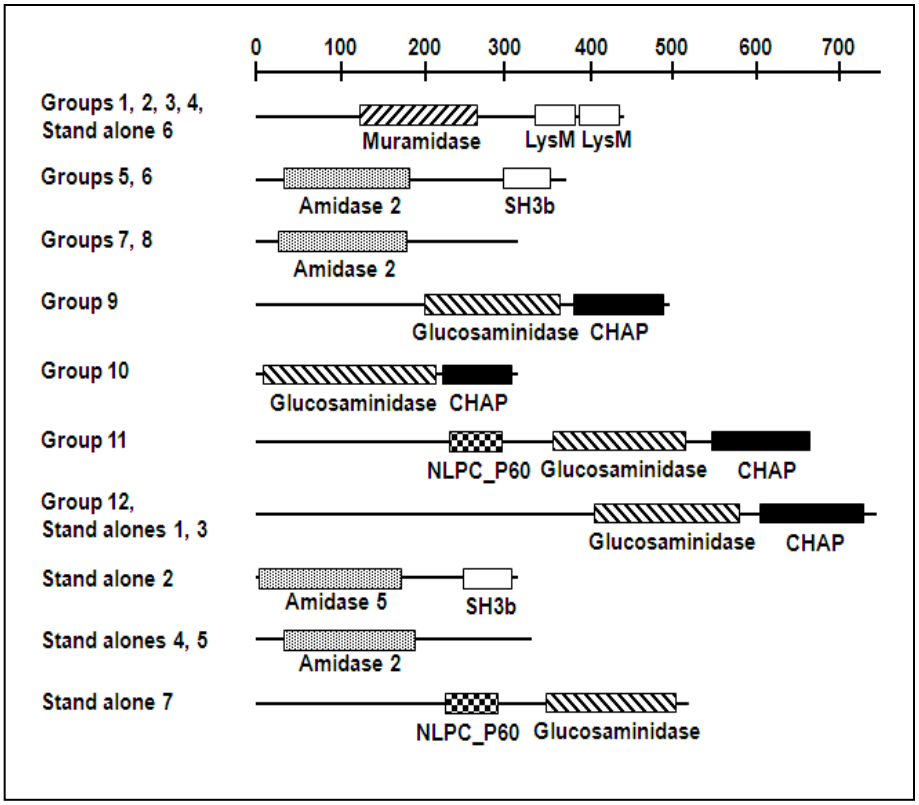
Muramidase
Stand alone 3
x1
+
x8
CHAP

Figure 5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Amidase 2
SH3b
Groups 7, 8
Amidase 2
Group 9
Glucosaminidase CHAP
Group 10
Glucosaminidase CHAP
Group 11
NLPC_P60 Glucosaminidase CHAP
Group 12,
Stand alones 1, 3
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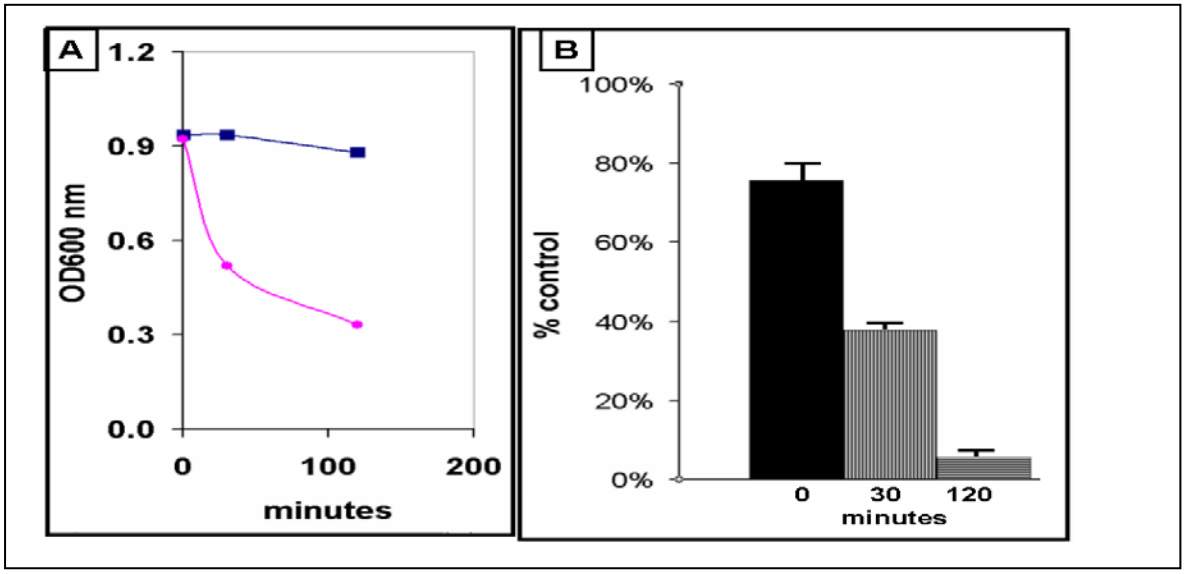
Amidase 2
Stand alone 7
NLPC_P60 Glucosaminidase

Figure 6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% control
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120
minutes

Table 1. Enterococcal lysins

	AA	ACCESION #
GROUP 1		
endolysin, putative [E. faecalis V583]	433	NP_814147.1
endolysin [E. faecalis ATCC 29200]	433	ZP_04437810.1
lysin [E. faecalis DS5]	433	ZP_05562195.1
lysin [E. faecalis T1]	433	ZP_05423767.1
lysin [E. faecalis HIP11704]	433	ZP_05568662.1
endolysin [phage phiFL4A]	433	YP_003347409.1
endolysin [E. faecalis V583]	433	NP_816427.1
lysin [E. faecalis AR01/DG]	433	ZP_05593964.1
endolysin [E. faecalis X98]	433	ZP_05598729.1
endolysin [phage phiFL1A]	433	YP_003347517.1
endolysin [phage phiFL2A]	433	YP_003347352.1
endolysin [phage phiFL1B]	433	ACZ63822.1
endolysin [phage phiFL1C]	433	ACZ63895.1
endolysin [phage phiFL2B]	433	ACZ64018.1
endolysin [E. faecalis T8]	433	ZP_05558876.1
lysin [E. faecalis JH1]	433	ZP_05573731.1
GROUP 2		
lysin [E. faecalis Merz96]	419	ZP_05565596.1
endolysin [E. faecalis R712]	419	ZP_06629599.1
endolysin [E. faecalis S613]	419	ZP_06631635.1
endolysin [phage phiEf11]	419	YP_003358816.1
endolysin [E. faecalis X98]	419	ZP_05599066.1
endolysin [E. faecalis CH188]	419	ZP_05585395.1
endolysin [phage phiFL3A]	419	YP_003347625.1
endolysin [phage phiFL3B]	419	ACZ64148.1
lysin [E. faecalis JH1]	419	ZP_05572412.1
lysin [E. faecalis D6]	419	ZP_05581557.1
GROUP 3		
endolysin [E. faecalis ATCC 29200]	412	ZP_04438395.1
phage lysin [E. faecalis T1]	412	ZP_05422953.1
endolysin [E. faecalis V583]	413	NP_815667.1
phage lysin [E. faecalis HIP11704]	413	ZP_05568908.1
phage lysin [E. faecalis E1Sol]	413	ZP_05576004.1
endolysin [E. faecalis TX1322]	413	ZP_04434151.1
endolysin [E. faecalis CH188]	413	ZP_05584633.1
phage lysin [E. faecalis ATCC 4200] [1]	413	ZP_05476312.1
endolysin [E. faecalis TUSoD Ef11]	394	ZP_04647652.1
endolysin [E. faecalis T8]	413	ZP_05559457.1
GROUP 4		
endolysin [E. faecium E1039]	394	ZP_06675756.1
endolysin [E. faecium E1039]	425	ZP_06674744.1
GROUP 5		
PlyP100 [E. faecalis HIP11704]	322	ZP_05566775.1
endolysin [E. faecalis Merz96]	322	ZP_05564324.1
endolysin [E. faecalis R712]	368	ZP_06628454.1
endolysin [E. faecalis S613]	368	ZP_06632418.1
endolysin [E. faecalis DS5]	322	ZP_05561234.1
endolysin [E. faecalis T8]	351	ZP_05557995.1
endolysin [E. faecalis V583]	368	NP_815207.1
endolysin [E. faecalis R712]	368	ZP_06628239.1
endolysin [E. faecalis S613]	368	ZP_06633896.1
endolysin [E. faecalis Fly1]	341	ZP_05579618.1
GROUP 6		
amidase [E. faecalis TX0104]	374	ZP_03948603.1
amidase [E. faecalis HH22]	374	ZP_03983131.1
amidase [E. faecalis TX1322]	374	ZP_04434756.1
endolysin [E. faecalis R712]	374	ZP_06629056.1
endolysin [E. faecalis S613]	374	ZP_06632253.1
endolysin [E. faecalis V583]	365	NP_815016.1
endolysin [E. faecalis ATCC 29200]	374	ZP_04438946.1
endolysin [E. faecalis TUSoD Ef11]	365	ZP_04647840.1
endolysin [E. faecalis X98]	365	ZP_05599811.1
endolysin [E. faecalis T8]	361	ZP_05558304.1
endolysin [E. faecalis ATCC 4200]	352	ZP_05475717.1
endolysin [E. faecalis JH1]	350	ZP_05573170.1
endolysin [E. faecalis HIP11704]	345	ZP_05569483.1
endolysin [E. faecalis Fly1]	345	ZP_05579809.1
endolysin [E. faecalis Merz96]	345	ZP_05566285.1
endolysin [E. faecalis AR01/DG]	345	ZP_05592904.1
endolysin [E. faecalis DS5]	345	ZP_05562950.1
GROUP 7		
amidase [E. faecium 1,141,733]	338	ZP_05666679.1
amidase [E. faecium Com15]	339	ZP_05677833.1
amidase [E. faecium 1,231,501]	338	ZP_05664801.1
amidase [E. faecium E980]	339	ZP_06681905.1
amidase [E. faecium 1,230,933]	339	ZP_05659803.1
amidase [E. faecium U0317]	339	ZP_06702043.1
amidase [E. faecium 1,231,408]	339	ZP_05673558.1
amidase [E. faecium Com15]	338	ZP_05678707.1
amidase [E. faecium 1,231,410]	339	ZP_05671179.1
amidase [E. faecium E980]	336	ZP_06683607.1
amidase [E. faecium E1071]	339	ZP_06680220.1
amidase, family 2 [E. faecium C68]	320	ZP_05832333.1
amidase [E. faecium 1,230,933]	336	ZP_05659231.1
amidase [E. faecium 1,231,502]	336	ZP_05662248.1
amidase [E. faecium U0317]	336	ZP_06700224.1
amidase [E. faecium 1,231,501]	338	ZP_05663923.1
amidase [E. faecium 1,231,410]	321	ZP_05671689.1
amidase, family 2 [E. faecium TC 6]	323	ZP_05924003.1
amidase, family 2 [E. faecium D344SRF]	323	ZP_06447215.1
amidase [E. faecium 1,231,502]	306	ZP_05663252.1
amidase [E. faecium E1636]	308	ZP_06695864.1
GROUP 8		
amidase, family 2 [E. faecium DO]	341	ZP_00602919.1
amidase [E. faecium E1162]	341	ZP_06676885.1
amidase [E. faecium 1,231,408]	341	ZP_05673081.1
amidase [E. faecium 1,231,410]	323	ZP_05671663.1
amidase, family 2 [E. faecium C68]	322	ZP_05833245.1
amidase [E. faecium E1636]	310	ZP_06694650.1
amidase [E. faecium 1,231,502]	291	ZP_05661451.1
GROUP 9		
amidase [E. faecalis V583]	503	NP_814047.1
amidase [E. faecalis HH22]	503	ZP_03985946.1
amidase [E. faecalis T11]	503	ZP_05595649.1
amidase [E. faecalis Fly1]	503	ZP_05578550.1
amidase [E. faecalis TX0104]	503	ZP_03950088.1
amidase [E. faecalis AR01/DG]	503	ZP_05594613.1
amidase [E. faecalis Merz96]	503	ZP_05564795.1
amidase, family 4 [E. faecalis R712]	503	ZP_06628637.1
amidase, family 4 [E. faecalis S613]	503	ZP_06632633.1
amidase, family 4 [E. faecalis T8]	503	ZP_05560568.1
amidase [E. faecalis HIP11704]	503	ZP_05568347.1
amidase [E. faecalis ATCC 4200]	503	ZP_05475182.1
amidase [E. faecalis TX1322]	503	ZP_04435643.1
amidase [E. faecalis X98]	503	ZP_05598533.1
amidase [E. faecalis ATCC 29200]	501	ZP_04439231.1
amidase [E. faecalis DS5]	503	ZP_05560989.1
amidase [E. faecalis E1Sol]	503	ZP_05575902.1
amidase [E. faecalis JH1]	503	ZP_05572849.1
amidase [E. faecalis TUSoD Ef11]	501	ZP_04648145.1
GROUP 10		
amidase [E. faecalis TX0104]	309	ZP_03948310.1
amidase, family 4 [E. faecalis R712]	309	ZP_06630528.1
amidase, family 4 [E. faecalis S613]	309	ZP_06633335.1
GROUP 11		
amidase [E. faecalis T1]	663	ZP_05423074.1
amidase [E. faecalis T11]	649	ZP_05596538.1
amidase [E. faecalis Fly1]	652	ZP_05579285.1
amidase [E. faecalis E1Sol]	649	ZP_05576670.1
amidase [E. faecalis V583]	652	NP_815520.1
amidase [E. faecalis TX0104]	652	ZP_03949059.1
amidase [E. faecalis HH22]	652	ZP_03983681.1
amidase, family 4 [E. faecalis R712]	652	ZP_06629298.1
amidase, family 4 [E. faecalis S613]	652	ZP_06633447.1
GROUP 12		
amidase [E. casseliflavus EC20]	655	ZP_05655421.1
amidase [E. casseliflavus EC30]	650	ZP_05645789.1
amidase [E. casseliflavus EC10]	650	ZP_05652119.1
STAND ALONE PROTEINS		
1 amidase [E. gallinarum EG2]	703	ZP_05649621.1
2 PlyV12 [phage phi1]	314	AAT01859.1
3 amidase [E. casseliflavus EC20]	715	ZP_05656866.1
4 amidase [phage phiEF24C]	289	YP_001504118.1
5 amidase [phage EFAP-1]	328	YP_002727874.1
6 endolysin [E. faecalis HH22]	270	ZP_03985506.1
7 amidase [E. faecalis T3]	523	ZP_05503383.1

Identities within groups are generally ≥ 90%.
Exception: [1] 89%;

Table 2. Staphylococcal lysins

	AA	ACCESSION #
GROUP 1		
putative lysin [Staphylococcus phage K]	495	YP_024461
endolysin [Staphylococcus phage 812]	494	ABL87139
endolysin [Staphylococcus phage GH15]	495	ADG26756
GROUP 2		
N-acetylmuramoyl-L-alanine amidase [S. epidermidis M23864:W2(grey)]	487	ZP_06612943
autolysin (N-acetylmuramoyl-L-alanine amidase) [S. caprae C87]	487	ZP_07841306
autolysin (N-acetylmuramoyl-L-alanine amidase) [S. capitis SK14]	487	ZP_03614343
GROUP 3		
amidase [Staphylococcus phage 44AHJD]	250	NP_817310
ORF009 [Staphylococcus phage 66]	250	YP_239469
amidase [Staphylococcus phage SAP-2]	249	YP_001491539
GROUP 4		
lytic enzyme [S. aureus subsp. aureus N315]	251	NP_375054
autolysin [S. aureus subsp. aureus MR1]	251	ZP_06859751
lytic enzyme [S. aureus subsp. aureus MW2]	251	NP_646703
autolysin [S. aureus subsp. aureus MSSA476]	251	YP_043983
gametolysin [S. aureus subsp. aureus A017934/97]	251	ZP_06376153
N-acetylmuramoyl-L-alanine amidase [S. aureus subsp. aureus H19]	251	ZP_06343995
lytic enzyme (N-acetylmuramoyl-L-alanine amidase) [Staphylococcus prophage phiPV83]	251	NP_061648
ORF017 [Staphylococcus phage 42E]	251	YP_239884
GROUP 5		
hypothetical protein 44AHJD_11 [Staphylococcus phage 44AHJD]	479	NP_817306
ORF004 [Staphylococcus phage 66]	487	YP_239474
hypothetical protein SAP2_gp10 [Staphylococcus phage SAP-2] [1]	478	YP_001491535
GROUP 6		
amidase [Staphylococcus phage phi2958PVL]	484	YP_002268027
amidase (peptidoglycan hydrolase) [Staphylococcus phage PVL]	484	NP_058463
amidase [Staphylococcus phage tp310-1]	484	YP_001429893
truncated amidase [S. aureus subsp. aureus MW2]	484	NP_646197
amidase [S. aureus A6224]	484	ZP_05696927
ORF006 [Staphylococcus phage 96]	484	YP_240259
prophage amidase, putative [S. aureus subsp. aureus ED133]	484	ADI96879
putative amidase [S. aureus subsp. aureus ED98]	484	YP_003282866
amidase [Staphylococcus phage phiSLT]	484	NP_075522
amidase [S. aureus subsp. aureus ST398]	484	CAQ48834
77ORF005 [Staphylococcus phage 77]	484	NP_958622
amidase [S. aureus subsp. aureus MRSA252]	484	YP_040898
prophage L54a, amidase, putative [S. aureus subsp. aureus COL]	484	YP_185281
prophage L54a, amidase, putative [S. aureus subsp. aureus CGS03]	484	EFT84462
amidase [Staphylococcus phage tp310-2]	484	YP_001429961
amidase [S. aureus subsp. aureus MSSA476]	484	YP_043081
putative endolysin [Staphylococcus phage phiSauS-IPLA35]	484	YP_002332423
N-acetylmuramoyl-L-alanine amidase [S. aureus A10102]	484	ZP_06334988
peptidoglycan hydrolase [Staphylococcus phage phi12]	484	NP_803355
N-acetylmuramoyl-L-alanine amidase [ORF007 Staphylococcus phage 47]	484	%YP_240025
peptidoglycan hydrolase, putative [S. aureus subsp. aureus 132]	484	ZP_06378887
amidase [S. aureus A6300]	484	ZP_05693770
N-acetylmuramoyl-L-alanine amidase [S. aureus A9765]	484	ZP_06329456
amidase [S. aureus subsp. aureus 65-1322]	484	ZP_05604610
ORF008 [Staphylococcus phage 3A]	484	YP_239959
GROUP 7		
amidase [Staphylococcus phage CNPH82]	460	YP_950628
phage amidase [Staphylococcus phage PH15]	460	YP_950690
bacteriophage amidase [S. epidermidis M23864:W1] [2]	460	ZP_04819028
GROUP 8		
CHAP domain-containing protein [S. aureus subsp. aureus JH9]	470	YP_001246290
bacteriophage amidase [S. aureus subsp. aureus USA300_TCH959]	473	ZP_04865682
phage amidase [S. aureus subsp. aureus 132]	470	ZP_06378624
phage amidase [S. aureus subsp. aureus MR1]	470	ZP_06859762
phage amidase [S. aureus subsp. aureus ED98]	470	YP_003281797
CHAP domain-containing protein [S. aureus A6300]	470	ZP_05694219
similar to phage phi PVL amidase [Staphylococcus phage phiETA]	470	NP_510959
amidase [Staphylococcus phage phiETA2]	470	YP_001004328
amidase [Staphylococcus phage phiETA3]	470	YP_001004396
ORF007 [Staphylococcus phage 71]	470	YP_240407
GROUP 9		
Autolysin (S. aureus) [3]	481	LYTA_STAAU
amidase [Staphylococcus phage 80alpha] [4]	481	AAB39699
phage amidase [S. aureus subsp. aureus str. Newman]	481	YP_001332073
amidase [S. aureus A9719]	486	ZP_05684021
N-acetylmuramoyl-L-alanine amidase [S. aureus subsp. aureus D139]	484	ZP_06324909
N-acetylmuramoyl-L-alanine amidase [S. aureus A9765]	484	ZP_06327634
ORF007 [Staphylococcus phage 29]	481	YP_240560
autolysin [S. aureus subsp. aureus NCTC 8325]	481	YP_500516
Autolysin, hypothetical phage protein [S. aureus subsp. aureus TW20]	481	CBI48272
amidase [S. aureus subsp. aureus Mu50]	481	NP_371437
ORF006 [Staphylococcus phage 88]	481	YP_240699
endolysin [Staphylococcus phage phiMR11]	481	YP_001604156
putative cell wall hydrolase [Staphylococcus phage phiMR25]	481	YP_001949866
N-acetylmuramoyl-L-alanine amidase [S. aureus subsp. aureus C427]	484	ZP_06327377
N-acetylmuramoyl-L-alanine amidase [S. aureus subsp. aureus JH9]	481	YP_001246457
ORF007 [Staphylococcus phage 55]	481	YP_240484
N-acetylmuramoyl-L-alanine amidase [S. aureus A6300]	486	ZP_05693156
ORF007 [Staphylococcus phage 69]	481	YP_239596
ORF007 [Staphylococcus phage 52A]	481	YP_240634
N-acetylmuramoyl-L-alanine amidase [S. aureus subsp. aureus MN8]	481	ZP_06948777
ORF006 [Staphylococcus phage 92]	481	YP_240773
autolysin [S. aureus subsp. aureus JKD6009]	481	ZP_03566881
phage amidase [S. aureus A9635]	484	ZP_05687279
phage-related amidase [S. aureus subsp. aureus CGS00]	481	EFU23738
Autolysin (N-acetylmuramoyl-L-alanine amidase) [S. aureus subsp. aureus ST398]	481	CAQ49916
endolysin [Staphylococcus phage phiSauS-IPLA88]	486	YP_002332536
GROUP 10		
cell wall hydrolase [Staphylococcus phage 11]	632	NP_803302
ORF004 [Staphylococcus phage 69]	632	YP_239591
cell wall hydrolase [Staphylococcus phage phiNM]	632	YP_874009
cell wall hydrolase [Staphylococcus phage TEM126]	632	ADV76510
autolysin [S. aureus A9765]	632	ZP_06327630
mannosyl-glycoprotein endo-beta-N-acetylglucosaminidase [S. aureus subsp. aureus JH9]	632	YP_001246286
mannosyl-glycoprotein endo-beta-N-acetylglucosaminidase [S. aureus A8115]	632	ZP_05690673
mannosyl-glycoprotein endo-beta-N-acetylglucosaminidase [S. aureus subsp. aureus CGS03]	589	EFT84342
phage N-acetylglucosamidase [S. aureus subsp. aureus CGS00]	632	EFU23742
ORF004 [Staphylococcus phage 85]	632	YP_239746
phage N-acetylglucosamidase [S. aureus subsp. aureus str. Newman]	632	YP_001331343
cell wall hydrolase [S. aureus subsp. aureus Mu50]	632	NP_371433
cell wall hydrolase [Staphylococcus phage phiETA2]	632	YP_001004324
cell wall hydrolase [Staphylococcus phage SAP-26]	632	YP_003857090
putative tail-associated cell wall hydrolase [Staphylococcus phage phiMR25]	632	YP_001949862
mannosyl-glycoprotein endo-beta-N-acetylglucosaminidase [S. aureus subsp. aureus D139]	632	ZP_06324913
mannosyl-glycoprotein endo-beta-N-acetylglucosaminidase [S. aureus subsp. aureus C427]	632	ZP_06327381
lyz [Staphylococcus phage 80alpha]	632	YP_001285381
ORF004 [Staphylococcus phage 53]	632	YP_239671
phage-related cell wall hydrolase [S. aureus RF122] [5]	634	YP_417168
putative peptidoglycan hydrolase [Staphylococcus phage phiSauS-IPLA88] [6]	634	YP_002332533
GROUP 11		
ORF004 [Staphylococcus phage 71]	624	YP_240403
similar to phage phi187 cell hydrolase Ply187 [Staphylococcus phage phiETA]	624	NP_510955
mannosyl-glycoprotein endo-beta-N-acetylglucosaminidase [S. aureus subsp. aureus 132]	624	ZP_06378620
mannosyl-glycoprotein endo-beta-N-acetylglucosaminidase [S. aureus subsp. aureus str. CF-Marseille]	624	ZP_04837774
conserved hypothetical protein [S. aureus A9635]	624	ZP_05687283
ORF004 [Staphylococcus phage 55]	624	YP_240479
cell wall hydrolase [Staphylococcus phage phiETA3]	624	YP_001004392
tail tip protein [Staphylococcus phage phiMR11]	624	YP_001604152
ORF004 [Staphylococcus phage ROSA]	624	YP_240329
ORF004 [Staphylococcus phage 96]	624	YP_240255
ORF004 [Staphylococcus phage 88]	624	YP_240695
ORF004 [Staphylococcus phage 29]	624	YP_240556
ORF005 [Staphylococcus phage X2]	624	YP_240843
mannosyl-glycoprotein endo-beta-N-acetylglucosaminidase [S. aureus subsp. aureus JKD6009]	624	ZP_03566885
hypothetical protein HMPREF0776_1895 [S. aureus subsp. aureus USA300_TCH959]	624	ZP_04865678
mannosyl-glycoprotein endo-beta-N-acetylglucosaminidase [S. aureus subsp. aureus H19]	624	ZP_06343859
GROUP 12		
hydrolase [Staphylococcus phage PH15] [7]	633	YP_950686

hydrolase [S. epidermidis BCM-HMP0060] [8]	607	ZP_04824942
amidase [Staphylococcus phage CNPH82]	633	YP_950623
N-acetylmuramoyl-L-alanine amidase [S. epidermidis M23864:W2(grey)] [9]	635	ZP_06614671
GROUP 13		
bifunctional autolysin Atl/ N-acetylmuramoyl-L-alanine amidase/ endo-beta-N-acetylglucosaminidase [S. pseudintermedius HKU10-03] [10]	629	YP_004148762
ORF002 [Staphylococcus phage 187]	628	YP_239513
cell wall hydrolase Ply187 [Staphylococcus phage 187]	628	CAA69022
Stand alone proteins		
1 Lysostaphin [S. simulans]	389	AAA26655
2 Endolysin [Staphylococcus phage 812]	284	ABL87142
3 Lytic enzyme, amidase [S. aureus]	426	ACZ59017
4 Endolysin [Staphylococcus phageSA4]	267	ADR02788
5 Glycyl-glycine endopeptidase ALE1	362	ALE1-STACP
6 Lysine [Bacteriophage phi WMY]	477	BAD83402
7 Phage amidase [Staphylococcus aureus subsp. aureus TW20]	500	CBI50050
8 lysostaphin	480	LSTP_STAST
9 Phage N-acetylmuramoyl-L-alanine amidase [S. lugdunensis HKU09-01]	488	YP_003472450
10 lysostaphin [S. simulans bv. staphylolyticus]	452	YP_003505772
11 Autolysin [S. pseudintermedius HKU10-03]	251	YP_004148764
12 prophage, amidase, putative [S. epidermidis RP62A]	463	YP_189215
13 ORF015 [Staphylococcus phage Twort]	467	YP_238716
14 ORF021 [Staphylococcus phage 85]	213	YP_239752
15 ORF018 [Staphylococcus phage 85]	237	YP_239755
16 ORF007 [Staphylococcus phage 2638A]	486	YP_239818
17 ORF004 [Staphylococcus phage 37]	639	YP_240099
18 ORF006 [Staphylococcus phage 37]	481	YP_240103
19 ORF003 [Staphylococcus phage EW]	630	YP_240176
20 ORF007 [Staphylococcus phage EW]	482	YP_240182
21 ORF018 [Staphylococcus phage X2]	213	YP_240847
22 ORF019 [Staphylococcus phage X2]	210	YP_240849
23 amidase (peptidoglycan hydrolase) [S. haemolyticus JCSC1435]	464	YP_253663
24 N-acetylmuramoyl-L-alanine amidase [S. haemolyticus JCSC1435]	494	YP_254248
25 hypothetical protein SH2336 [S. haemolyticus JCSC1435]	647	YP_254251
26 mannosyl-glycoprotein endo-beta-N-acetylglucosaminidase [S. capitis SK14]	626	ZP_03614366
27 autolysin [S. warneri L37603]	477	ZP_04679079
28 possible N-acetylmuramoyl-L-alanine amidase [S. epidermidis BCM-HMP0060]	574	ZP_04824947
29 conserved hypothetical protein [S. aureus subsp. aureus E1410]	325	ZP_05610313
30 peptidoglycan hydrolase [S. aureus A9299]	405	ZP_05688267
31 amidase [S. aureus A9299]	405	ZP_05688584
32 conserved hypothetical protein [S. aureus A6300]	494	ZP_05694215
33 bacteriophage amidase [S. epidermidis M23864:W2(grey)]	467	ZP_06614678
34 N-acetylmuramoyl-L-alanine amidase [S. aureus A8819]	394	ZP_06817547
35 petidoglycan hydrolase, putative [S. aureus subsp. aureus MR1]	392	ZP_06859771
36 N-acetylmuramoyl-L-alanine amidase [S. aureus A8796]	419	ZP_06930779
37 N-acetylmuramoyl-L-alanine amidase [S. aureus subsp. aureus ATCC BAA-39]	564	ZP_07361756

Identities within groups are generally ≥ 90%. Exceptions: [1] 89%; [2] 87%; [3] 89%; [4] 89%; [5] 88%; [6] 88%; [7] 89%; [8] 87%; [9] 86%; [10] 84%;

Table 3. Streptococcal lysins

	AA	ACCESSION #
GROUP 1 a		
Cpl-1 [S. pneumoniae]	339	NP_044837.1
Cpl-9 [S. pneumoniae]	339	P19386.1
GROUP 1 b		
PH10 lysin [S. oralis]	334	YP_002925184.1
GROUP 1 c		
Cpl-7 [S. pneumoniae]	342	P19385.1
GROUP 2 a		
autolysin [S. pneumoniae SP3-BS71]	318	ZP_01819152.1
lytic amidase [S. pneumoniae SP195]	318	ZP_02714370.1
autolysin [S. pneumoniae SP11-BS70]	318	ZP_01824138.1
lytic amidase [S. pneumoniae CDC1873-00]	318	ZP_02708645.1
autolysin [S. pneumoniae SP19-BS75]	318	ZP_01832999.1
lytic amidase [S. pneumoniae 670-6B]	318	YP_003880285.1
lytic amidase [S. pneumoniae Hungary19A-6]	318	YP_001693491.1
autolysin [S. pneumoniae SP6-BS73]	318	ZP_01821560.1
autolysin [S. pneumoniae AP200]	318	YP_003875665.1
MM1 lysin [S. pneumoniae]	318	NP_150182.1
lytic amidase [S. pneumoniae SP195]	318	ZP_02712971.1
VO1 amidase [S. pneumoniae]	318	CAD35393.1
HB-3 amidase [S. pneumoniae]	318	P32762.1
lytic amidase [S. pneumoniae CDC3059-06]	318	ZP_02718952.1
lytic amidase [S. pneumoniae 70585]	318	YP_002739391.1
lytic amidase [S. pneumoniae SP-BS293]	318	ZP_07345341.1
lytic amidase [S. pneumoniae P1031]	318	YP_002737318.1
autolysin [S. pneumoniae SP23-BS72]	318	ZP_01835850.1
GROUP2 b		
autolysin [S. pneumoniae]	313	AAK29073.1
autolysin [S. pneumoniae TIGR4]	318	NP_346365.1
amidase [S. pneumoniae R6]	318	NP_359346.1
putative amidase [S. pneumoniae INV104]	318	CBW37351.1
autolysin [S. pneumoniae SP3-BS71]	318	ZP_01818711.1
VO1 amidase [S. pneumoniae 8249]	318	CAD35389.1
LytA amidase [S. pneumoniae]	318	CAJ34409.1
LytA amidase [S. pneumoniae]	318	CAJ34410.1
autolysin [S. pneumoniae 670-6B]	318	YP_003880176.1
autolysin [S. pneumoniae]	313	AAK29074.1
autolysin [S. pneumoniae CDC1087-00]	318	ZP_02711922.1
Autolysin [S. pneumoniae]	313	CBE65469.1
LytA autolysin [S. pneumoniae]	302	CAB53774.1
Autolysin [S. pneumoniae SP11-BS70]	318	ZP_01825916.1
LytA autolysin [S. pneumoniae]	302	CAB53770
autolysin [S. pneumoniae 670-6B]	318	YP_003878279.1
autolysin [S. pneumoniae SP14-BS69]	318	ZP_01828965.1
autolysin [S. pneumoniae JJA]	318	YP_002736862.1
GROUP 2 c		
LytA amidase [s. pneumoniae]	316	CAD12111.1
amidase [S. mitis SK597]	316	ZP_07640915.1
LytA amidase [s. pneumoniae]	316	CAD12115.1
LytA amidase [S. pneumoniae sp. 1504]	316	CAJ34416.1
LytA amidase [s. pneumoniae]	316	CAD12112.1
LytA amidase [S. pneumoniae]	316	CAD12116.1
LytA amidase [S. pneumoniae]	316	CAD12106.1
LytA amidase [S. pseudopneumoniae]	316	CAJ34411.1
LytA amidase [S. pneumoniae]	316	CAD12108.1
LytA amidase [S. pneumoniae sp. 578]	316	CAJ34413.1
LytA amidase [S. pneumoniae sp. 3072]	316	CAJ34420.1
LytA amidase [S. pneumoniae]	316	CAD12113.1
LytA amidase [S. pneumoniae]	316	CAD12110.1
LytA amidase [S. pneumoniae sp. 2410]	316	CAJ34419.1
LytA101 [S. pneumoniae]	316	AAB23082.1
Autolysin [S. mitis]	300	CAB76388.1
Autolysin [Streptococcus sp.]	300	CAB76391.1
LytA amidase [S. pneumoniae]	316	CAD12114.1
Autolysin [Streptococcus sp.]	300	CAB76389.1
Autolysin [Streptococcus sp.]	300	CAB76392.1
LytA amidase [S. pneumoniae sp. 1237]	316	CAJ34414.1
Autolysin [Streptococcus sp.]	300	CAB76394.1
LytA amidase [S. pneumoniae]	316	CAD12109.1
LytA amidase [S. pneumoniae]	316	CAD12107.2
Autolysin [Streptococcus sp.]	300	CAB76390.1
GROUP 2 d		
LytA amidase [S. mitis B6]	318	YP_003445618.1
LytA-like amidase [S. mitis]	318	CAF02035.1
EJ-1 lysin [S. pneumoniae]	316	NP_945312.1
GROUP 3 a		
putative lysin [S. pyogenes phage 315.2]	402	NP_664726.1
putative amidase [S. pyogenes phage 315.1]	401	NP_664535.1
phage-associated lysin [S. pyogenes NZ131]	402	YP_002286426.1
spyM18_0777 [S. pyogenes MGAS8232]	401	NP_606945.1
phage-associated lysin [Streptococcus phage 9429.1]	404	YP_596324.1
spyM18_1750 [S. pyogenes MGAS8232]	401	NP_607778.1
amidase [S. pyogenes MGAS10394]	401	YP_060660.1
putative phage amidase [S. pyogenes str. Manfredo]	401	YP_001128106.1
Spy_1438 [S. pyogenes M1 GAS]	401	NP_269522.1
spyM18_1448 [S. pyogenes MGAS8232]	401	NP_607527.1
amidase [S. pyogenes ATCC 10782]	401	ZP_07461342.1
Amidase [S. pyogenes ATCC10782]	401	ZP_07460525.1
GROUP 3 b		
315.4 lysin [S. pyogenes phage phiNIH1.1]	400	NP_438163.1
Phage associated lysin [S. pyogenes MGAS10394]	400	YP_059383.1
370.1 lysin [S. pyogenes]	400	NP_268942.1
amidase [S. pyogenes ATCC 10782]	400	ZP_07461599.1
lysin [S. dysgalactiae subsp. equisimilis GGS_124]	400	YP_002996819.1
P9 lysin [S. equi phage P9]	400	YP_001469230.1
GROUP 3 c		
315.6 lysin [S. pyogenes MGAS315]	244	NP_665215.1
SPs0453 [S. pyogenes SSI-1]	226	NP_801715.1
SPs1121 [S. pyogenes SSI-1]	226	NP_802383.1
GROUP 3 d		
phage-associated lysin [S. equi subsp. equi 4047]	404	YP_002745608.1
phage amidase [S. equi subsp. equi 4047]	403	YP_002746965.1
GROUP 3 e		
phage-associated lysin [S. pyogenes MGAS5005]	398	YP_282779.1
Phage 2096.1 lysin [group A Streptococcus]	398	YP_600196.1
Phage amidase [S. equi subsp. equi 4047] [1]	398	YP_002746181.1
GROUP 3 f		
spyM18_1242 [S. pyogenes MGAS8232]	161	NP_607353.1
GROUP 3 g		
Phage-associated lysin [S. pyogenes MGAS10394]	213	YP_060304.1
GROUP 4		
putative phage lysin [S. pyogenes phage 315.5]	254	NP_665110.1
SpyoM01000009 [S. pyogenes M49 591]	251	ZP_00366664.1
phage-associated lysin [S. pyogenes MGAS5005]	254	YP_282364.1
GROUP 5 a		
Phi3396 lysin [S. dysgalactiae subsp. equisimilis]	253	YP_001039943.1
Phage NZ131.2 lysin [S. pyogenes]	249	YP_002285797.1
Phage-associated lysin [S. pyogenes MGAS10394]	250	YP_060862.1
GROUP 5 b		
Phage-associated lysin [S. pyogenes MGAS10394]	203	YP_060515.1
GROUP 6 a		
phage 9429.2 lysin [S. pyogenes]	373	YP_596581.1
GROUP 6 b		
B30 lysin [S. agalactiae]	445	AAN28166.2
49.7 kDA protein [S. equi]	444	AAF72807.1
putative lysin [S. pyogenes phage 370.3]	444	NP_269184.1
PlyGBS [S. agalactiae phage NCTC11261]	443	AAR99416.1
phage-associated lysin [S. pyogenes MGAS6180]	444	YP_280438.1
prophage LambdaSa03 endolysin [S. agalactiae]	443	YP_329285.1
49.7 kDa protein [S. agalactiae 18RS21]	447	ZP_00780878.1
putative phage lysin [S. pyogenes strain Manfredo]	444	YP_001128574.1
phage lysin [S. equi subsp. equi 4047]	444	YP_002747253.1
GROUP 7		
LambdaSa1 lysin [S. agalactiae 2603V/R]	239	NP_687631.1
Endolysin [S. agalactiae H36B]	248	ZP_00782522.1
GROUP 8 a		
putative amidase [S. pyogenes phage 315.3]	404	NP_664900.1
putative amidase [S. pyogenes MGAS8232]	405	NP_606641.1
phage protein [S. pyogenes MGAS10750]	405	YP_602773.1
putative phage lysin [S. pyogenes str. Manfredo]	402	YP_001128256.1
GROUP 8 b		
LambdaSa2 lysin [S. dysgalactiae subsp. equisimilis GGS_124]	449	YP_002997317.1
GROUP 8 c		
LambdaSa2 lysin [S. agalactiae 2603V/R]	468	NP_688827.1
GROUP 8 d		
SMP lysin [S. suis]	481	YP_950557.1
GROUP 9 a		
Cell wall binding repeat family protein [S. mitis SK321]	568	ZP_07643272.1
Cell wall binding repeat family protein [S. mitis SK597]	570	ZP_07641594.1
endo-beta-N-acetylglucosaminidase [S. mitis NCTC 12261]	568	ZP_07645063.1
LytB [S. mitis]	568	ACO37163.1
LytB [S. mitis B6]	570	YP_003446078.1
GROUP 9 b		

endo-beta-N-acetylglucosaminidase [S. pneumoniae 70585]	702	YP_002740268.1
endo-beta-N-acetylglucosaminidase [S. pneumoniae G54]	702	YP_002037600.1
endo-beta-N-acetylglucosaminidase [S. pneumoniae Hungary19A-6]	702	YP_001694410.1
endo-beta-N-acetylglucosaminidase [S. pneumoniae P1031]	702	YP_002738134.1
endo-beta-N-acetylglucosaminidase [S. pneumoniae Taiwan19F-14]	702	YP_002742657.1
endo-beta-N-acetylglucosaminidase [S. pneumoniae BS397]	702	ZP_07350631.1
GROUP 9 c		
endo-beta-N-acetylglucosaminidase [S. pneumoniae SP-BS293]	614	ZP_07345852.1
endo-beta-N-acetylglucosaminidase [S. pneumoniae]	614	AAK19156.1
endo-beta-N-acetylglucosaminidase [S. pneumoniae CDC1087-00]	614	ZP_02710425.1
endo-beta-N-acetylglucosaminidase [S. pneumoniae INV104]	614	CBW36509.1
LytB [Spneumoniae AP200]	614	YP_003876588.1
GROUP 9 d		
endo-beta-N-acetylglucosaminidase [S. pneumoniae CGSP14]	677	YP_001835658.1
GROUP 9 e		
endo-beta-N-acetylglucosaminidase [S. pneumoniae CCRI 1974]	658	ZP_04525138.1
endo-beta-N-acetylglucosaminidase [S. pneumoniae CDC0288-04]	658	ZP_02715197.1
endo-beta-N-acetylglucosaminidase [S. pneumoniae CDC3059-06]	658	ZP_02718537.1
endo-beta-N-acetylglucosaminidase [S. pneumoniae JJA]	658	YP_002735981.1
endo-beta-N-acetylglucosaminidase [S. pneumoniae SP23-BS72]	658	ZP_01834875.1
endo-beta-N-acetylglucosaminidase [S. pneumoniae MLV-016]	658	ZP_02721563.1
endo-beta-N-acetylglucosaminidase [S. pneumoniae TIGR4]	658	NP_345446.1
endo-beta-N-acetylglucosaminidase [S. pneumoniae SP3-BS71]	658	ZP_01817975.1
GROUP 9 f		
endo-beta-N-acetylglucosaminidase [S. pneumoniae INV200]	721	CBW34519.1
endo-beta-N-acetylglucosaminidase [S. pneumoniae R6]	721	NP_358461.1
endo-beta-N-acetylglucosaminidase [S. pneumoniae TCH8431/19A]	721	YP_003724965.1
GROUP 10		
endo-beta-N-acetylglucosaminidase [S. mitis ATCC6249]	750	ZP_07462509.1
endo-beta-N-acetylglucosaminidase [S. sanguinis ATCC49296]	750	ZP_07887886.1
endo-beta-N-acetylglucosaminidase [Streptococcus sp. oral taxon str. 73H25AP]	750	ZP_07458768.1
GROUP 11 a		
Lysozyme [S. mitis NCTC 12261]	525	ZP_07644807.1
LytC Cpb13 [S. mitis B6]	536	YP_003446665.1
GROUP 11 b		
Cell wall binding protein [S. mitis SK564]	504	ZP_07642782.1
Cell wall binding protein [S. mitis SK597]	504	ZP_07641292.1
Cell wall binding protein [S. mitis SK321]	493	ZP_07642984.1
GROUP 11 c		
Lysozyme [S. pneumoniae SP3-BS71]	270	ZP_01818179.1
GROUP 11 d		
1,4-beta-N-acetylmuramidase [S. pneumoniae CDC1873-00]	490	ZP_02708500.1
1,4-beta-N-acetylmuramidase [S. pneumoniae P1031]	490	YP_002738710.1
1,4-beta-N-acetylmuramidase [S. pneumoniae SP11-BS70]	490	ZP_01824964.1
1,4-beta-N-acetylmuramidase [S. pneumoniae SP9-BS68]	490	ZP_01822918.1
1,4-beta-N-acetylmuramidase [S. pneumoniae 70585]	490	YP_002740840.1
1,4-beta-N-acetylmuramidase [S. pneumoniae CDC1087-00]	490	ZP_02711346.1
1,4-beta-N-acetylmuramidase [S. pneumoniae TCH8431/19A]	501	YP_003725251.1
1,4-beta-N-acetylmuramidase [S. pneumoniae R6]	501	NP_359024.1
1,4-beta-N-acetylmuramidase [S. pneumoniae]	492	AAK19157.1
1,4-beta-N-acetylmuramidase [S. pneumoniae SP18-BS74]	490	ZP_01831146.1
ATP dependent protease [S. pneumoniae SP23-BS72]	490	ZP_01836005.1
ATP dependent protease [S. pneumoniae SP6-BS73]	490	ZP_01820060.1
endo-beta-N-acetylglucosaminidase [S. pneumoniae G54]	490	YP_002038205.1
Lysozyme [S. pneumoniae Taiwan 19F-14]	493	YP_002742915.1
Lysozyme [S. pneumoniae BS455]	490	ZP_07341428.1
Lysozyme [S. pneumoniae CGSP14]	501	YP_001836276.1
LytC autolysin [S. pneumoniae]	501	CAA08765.1
Putative choline binding glycosyl hydrolase [S. pneumoniae INV104]	490	CBW37026.1
Putative choline binding glycosyl hydrolase [S. pneumoniae ATCC700669]	490	YP_002511487.1
SpneCMD 07616 [S. pneumoniae str. Canada MDR 19F]	490	ZP_06964203.1
SpneT 0200379 [S. pneumoniae TIGR4]	490	ZP_01409152.1
GROUP 11 e		
1,4-beta-N-acetylmuramidase [S. pneumoniae SP14-BS69]	311	ZP_01828088.1
GROUP 11 f		
Lysozyme [S. pneumoniae SP19-BS75]	227	ZP_01833670.1
GROUP 12 a		
Pal [S. pneumoniae phage DP-1]	296	O03979.1
GROUP 12 b		
gp56 [Streptococcus phage SM1]	295	NP_862895.1
GROUP 13 a		
S3b lysin [S. thermophilus] [2]	206 + 82 [5]	AAF24749.1
DT1 lysin [S. thermophilus]	200 + 75 [5]	NP_049413.1 + NP_049415.2
ALQ13.2 lysin [S. thermophilus]	200 + 75 [5]	YP_003344870.1+ YP_003344872.1
Orf28 [S. thermophilus phage 858]	200 + 75 [5]	YP_001686822.1+ YP_001686825.1
Phage 2972 lysin [S. thermophilus] [3]	199 + 75 [5]	YP_238509.1 + YP_238512.1
GROUP 13 b		
Putative phage PH15 endolysin [S. gordonii]	283	YP_001974380.1
GROUP 13 c		
Abc2 lysin [S. thermophilus]	281	YP_003347431.1
ORF44 [S. thermophilus phage 7201]	281	NP_038345.1
Phage 5093 lysin [S. thermophilus CSK939]	281	YP_002925118.1
Phage O1205 p51 [S. thermophilus CNRZ1205] [4]	281	NP_695129.1
GROUP 13 d		
Sfi11 lysin [S. thermophilus]	288	NP_056699.1
Sfi18 lysin [S. thermophilus]	288	AAF63073.1
Sfi19 lysin [S. thermophilus]	288	NP_049942.1
Sfi21 lysin [S. thermophilus]	288	NP_049985.1
GROUP 13 e		
STRINF 01560 [S. infantarius subsp. infantarius ATCC BAA-102]	281	ZP_02920679.1
STAND ALONE PROTEINS		
1 700P1 lysin [S. uberis]	236	ABB02702.1
2 Phage M102 gp19S [S. mutans]	273	YP_002995476.1
3 PlyC [Group A Streptococcus phage C1]	465 + 72 [6]	NP_852017.2

Identities within groups are generally ≥ 90%. Exceptions:
[1] 88%; [2] 88%; [3] 84%; [4] 86%;
[5] Encoded by two coding regions separated by an intron
[6] Multimeric protein consisting of two gene products

Table 4. Summary of in vivo studies with phage endolysin as antimicrobial.			
Bacteria	**Phage**	**Endolysin**	**Reference**
Streptococcus pneumoniae	Cp-1	Cpl-1	Loeffler *et al*., 2001 Loeffler *et al*., 2003 Loeffler & Fischetti, 2003 Jado *et al*., 2003 Entenza *et al*., 2005 McCullers *et al*., 2007 Grandgirard *et al*., 2008
Streptococcus pneumoniae	Dp-1	PAL	Loeffler & Fischetti, 2003 Jado *et al*., 2003
Streptococcus pyogenes	C1	C1*	Nelson *et al*., 2001
Streptococcus agalactiae	NCTC 11361	PlyGBS	Cheng *et al*., 2005
Bacillus anthracis	γ	PlyG	Schuch *et al*., 2002
	N/A**	PlyPH	Yoong *et al*., 2006
Staphylococcus aureus	MR11	MV-L	Rashel *et al*., 2007
	N/A***	ClyS	Daniel *et al*., 2010
	Bacteriophage K	$CHAP_k$	Fenton *et al*., 2010
	GH15	LysGH15	Gu *et al*., 2011

*Renamed PlyC according to (Nelson *et al.,* 2006)

**This endolysin was amplified from a prophage of the *Bacillus anthracis* Ames strain

***Chimeric construct from the bacteriophage Twort and PhiNM3 endolysins