CHAPTER 7

Endolysins as Antimicrobials

Daniel C. Nelson,[*,†] Mathias Schmelcher,[‡] Lorena Rodriguez-Rubio,[§] Jochen Klumpp,[‖] David G. Pritchard,[¶] Shengli Dong,[¶] and David M. Donovan[‡,1]

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[*] Institute for Bioscience and Biotechnology Research, University of Maryland, Rockville, Maryland, USA
[†] Department of Veterinary Medicine, Virginia-Maryland Regional College of Veterinary Medicine, College Park, Maryland, USA
[‡] Animal Biosciences and Biotechnology Laboratory, ANRI, ARS, USDA, Beltsville, Maryland, USA
[§] Instituto de Productos Lácteos de Asturias (IPLA-CSIC), Villaviciosa, Asturias, Spain
[‖] Institute of Food, Nutrition and Health, ETH Zurich, Zurich, Switzerland
[¶] Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, Alabama, USA
[1] Corresponding author, E-mail: david.donovan@ars.usda.gov

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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 (CBDs). 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 coevolution of phage and host, it is thought they are much less likely to invoke resistance. 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 phage endolysin work in a broader context, this chapter includes relevant studies of other well-characterized PG hydrolase antimicrobials.

ABBREVIATIONS:

CBD	cell wall-binding domain;
CFU	colony-forming unit;
CHAP	cysteine, histidine-dependent amidohydrolase/peptidase;
CPP	cell-penetrating peptides;
CSF	cerebrospinal fluid;
GlcNAc	*N*-acetylglucosamine;
HIV	Human Immunodeficiency Virus
HPLC	high-pressure liquid chromatography

IV	intravenous
MBC	minimum bactericidal concentration;
mDAP	*meso*-diaminopimelic acid;
MIC	minimum inhibitory concentration;
MRSA	methicillin-resistant *Staphylococcus aureus*;
MS	mass spectrometry;
MurNAc	*N*-acetylmuramic acid;
OD	optical density (ΔOD; change in OD);
PG	peptidoglycan;
PTD	protein transduction domain;
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis;
TAT	transactivator of transcription domain

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 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 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 exolysins is lysostaphin, a PG hydrolase secreted by *Staphylococcus simulans* that cleaves the *Staphylococcus 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.

Peptidoglycan 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 (1940). Work by Moak and Molineux (2004) demonstrated that PG hydrolases were associated with numerous phage particles infecting either Gram-negative or Gram-positive bacteria. These lytic structural proteins, which are mostly tail associated, cause localized degradation of the cell wall to enable infection of the bacterial host. Alternatively, phages 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), and 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 is discussed at the end of this chapter.

II. PEPTIDOGLYCAN STRUCTURE

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 a review, see Schleifer and Kandler (1972)]. The glycan polymer is in turn linked covalently 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 cross-linked 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 two L-alanines. 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

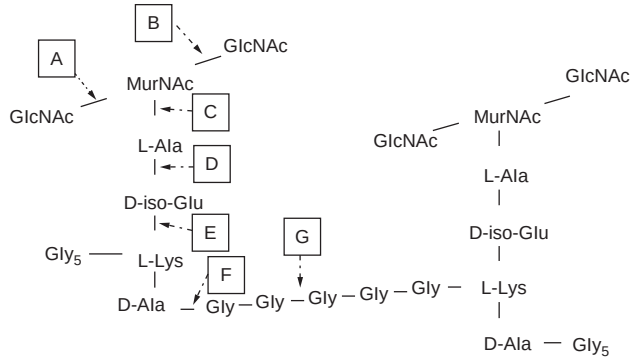

FIGURE 1 Structure of *Staphylococcus aureus* bacterial PG and cleavage sites by PG hydrolases. (A) An *N*-acetylglucosaminidase hydrolyzes the glycan component of the PG on the reducing side of GlcNAc. (B) In contrast, an *N*-acetylmuramidase (also known as "muramidase" or "lysozyme") hydrolyzes the glycan component of the PG on the reducing side of MurNAc. Likewise, lytic transglycosylases cleave the same bond, but form N-acetyl-1,6-anhydro-muramyl 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–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 that the structure of the *S. aureus* PG is depicted for illustration purposes. Other bacterial species have interpeptide bridges composed of different amino acids or may lack an interpeptide bridge altogether. In these organisms, a mDAP replaces L-Lys and directly cross-links to the terminal D-Ala of the opposite peptide chain.

the stem peptide instead of L-lysine. In these organisms, mDAP cross-links directly 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 cross-linking 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 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 found frequently 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 referred to commonly as a ''muramidase'' or ''lysozyme'' and is found frequently 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. 1 C) This activity is associated more often 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 favored evolutionarily by bacteriophages that require rapid lysis of host cells for the 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 (Figs. 1D–1 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 N-acetyl-1,6-anhydro-muramyl moiety residue during glycosidic cleavage and thus belong to a different mechanistic class than the lysozymes (Holtje and Tomasz, 1975). The phage Lambda endolysin (Taylor and Gorazdowska, 1974) and the gp144 endolysin from the ΦKZ

bacteriophage (Paradis-Bleau *et al.*, 2007) were both confirmed biochemically 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, 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 a 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 described originally by Satake *et al.* (1960) and modified by Mokrasch (1967). N-terminal sequencing of digestion products (i.e., Edman degradation) can also reveal cleavage sites of a PG hydrolase possessing endopeptidase activity (Navarre *et al.*, 1999; Pritchard *et al.*, 2004). Alternatively, digestion products can be labeled with 1-fluoro-2,4-dinitrobenzene, followed by HCl hydrolysis and reverse-phase high-pressure liquid chromatography (HPLC) (Fukushima *et al.*, 2007). HPLC peaks can be analyzed by mass spectrometry (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 earlier were used in an elegant series of experiments that showed that the streptococcal phage B30 endolysin contains both glycosidase and 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.

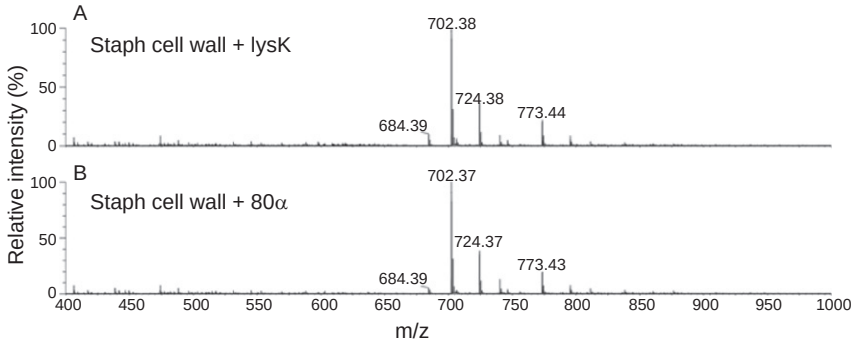

FIGURE 2 Electron spray ionization mass spectrometry determination of LysK and phi80α endolysin cut sites in *S. aureus* PG. Purified *S. aureus* PG was digested with LysK and phi80α endolysin under identical conditions as described in Becker *et al.* (2009). Digests were filtered through 5 K cutoff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

Another challenge is the generic classification of many endolysins simply as ''amidases,'' which is used ubiquitously 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 complicate this issue further, 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 that 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 that the streptococcal endolysins λSa1 and λSa2 contain *N*-acetylmuramoyl-L-alanine amidase activities. However, utilizing electrospray ionization mass spectrometry, Pritchard *et al.* (2007) 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. Clearly, more rigorous biochemical characterization of bacteriophage endolysins will help better define and predict the catalytic classes of these enzymes.

D. Endolysin modular structure

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. Most lysins from phage that infect Gram-negative hosts are single domain globular proteins typically composed 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) have been 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).

2. Gram-positive endolysin structure

In contrast to 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 cross-linked 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 phage endolysins and autolysins are the products of single genes, although 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 the C1 endolysin was later shown to be synthesized from two genes. This enzyme is composed of a gene product, PlyCA, that 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 multigene, heterononomer (nine subunit protein) are not abundantly clear. Nonetheless, nanogram quantities of PlyC can achieve $\approx$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 by Hermoso *et al.* (2007). A very complete discussion

of the PG hydrolase endopeptidase activities and their active site structure was also 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).

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 PG digestion (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 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, the authors have attempted to compile known PG hydrolase sequences for each of three genera—*Staphylococcus*, *Streptococcus*, and *Enterococcus*. These protein structures are collated in Figures 3–5. This summary sheds light on the degree of domain conservation and the range of lytic protein domain organization within and among these closely related genera. Within each genus, endolysins have been collated into groups based on protein architecture and sequence homology. Group members are listed in Tables I–III. 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 known to exist within each (Lindsay, 2008; Palmer *et al.*, 2010; Rossolini *et al.*, 2010). Each of the domains listed in Figures 3–5 can be found in public data sets describing conserved domains (PFAM: http://pfam.sanger.ac.uk/ or NCBI-conserved domain database: http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml).

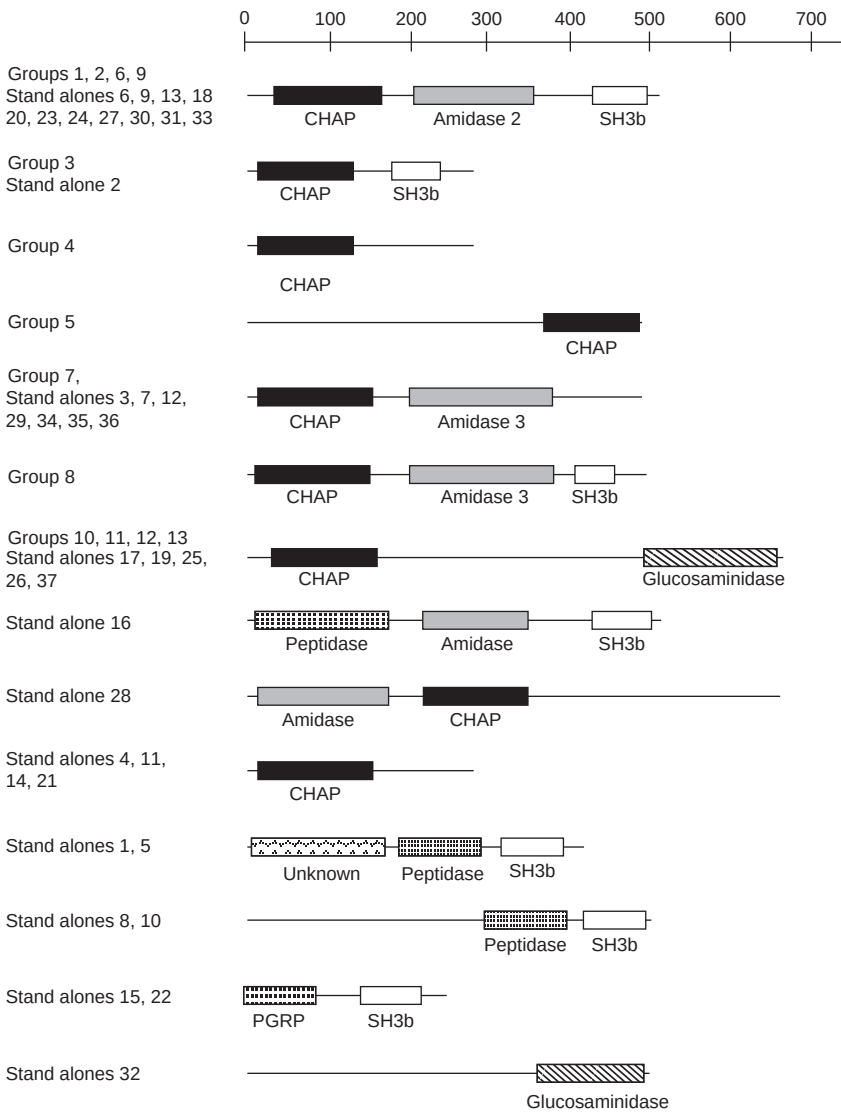

FIGURE 3 Staphylococcal PG hydrolase structure. Groups are derived from homology clustering performed in BLAST, NCBI of the proteins described in Table I. Scale bar represents number of amino acids. Domains are defined more clearly in the PFAM database http://www.sanger.ac.uk/resources/databases/pfam.html. White boxes represent CBDs. SH3b, bacterial Src homology 3 domain (Ponting *et al.*, 1999; Whisstock and Lesk, 1999); PGRP, peptidoglycan recognition protein (Dziarski and Gupta, 2006).

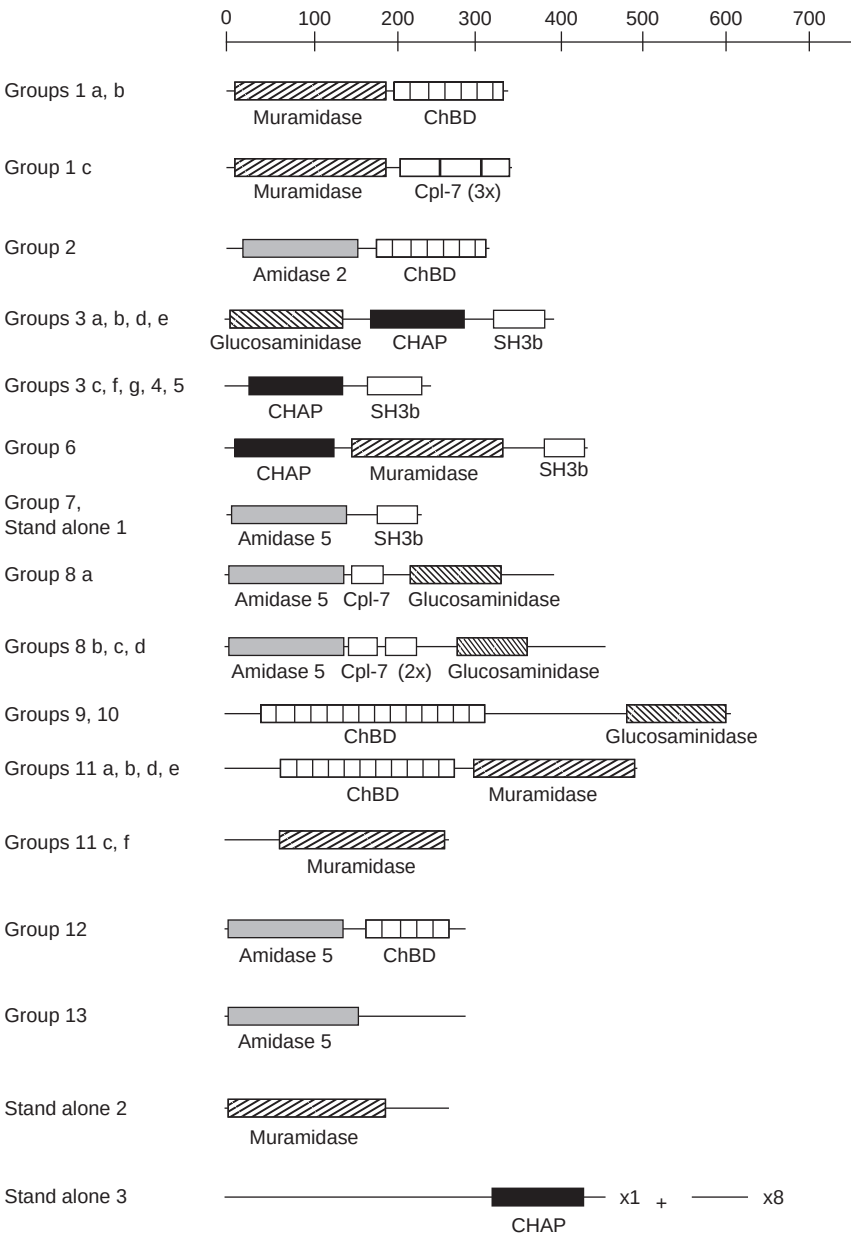

FIGURE 4 Streptococcal PG hydrolases. Groups are derived from homology clustering performed in BLAST, NCBI of the proteins described in Table II. Scale bar represents number of amino acids. Domains are defined more clearly in the PFAM database http://www.sanger.ac.uk/resources/databases/pfam.html. White boxes represent CBDs. ChBD, choline-binding domain (Hermoso *et al*., 2003); Cpl-7, cell wall-binding domain (Garcia *et al*., 1990); SH3b, bacterial Src homology 3 domain (Ponting *et al*., 1999; Whisstock and Lesk, 1999). Stand alone protein 3 is a multimeric lysin consisting of 1 big subunit and 8 copies of a small subunit.

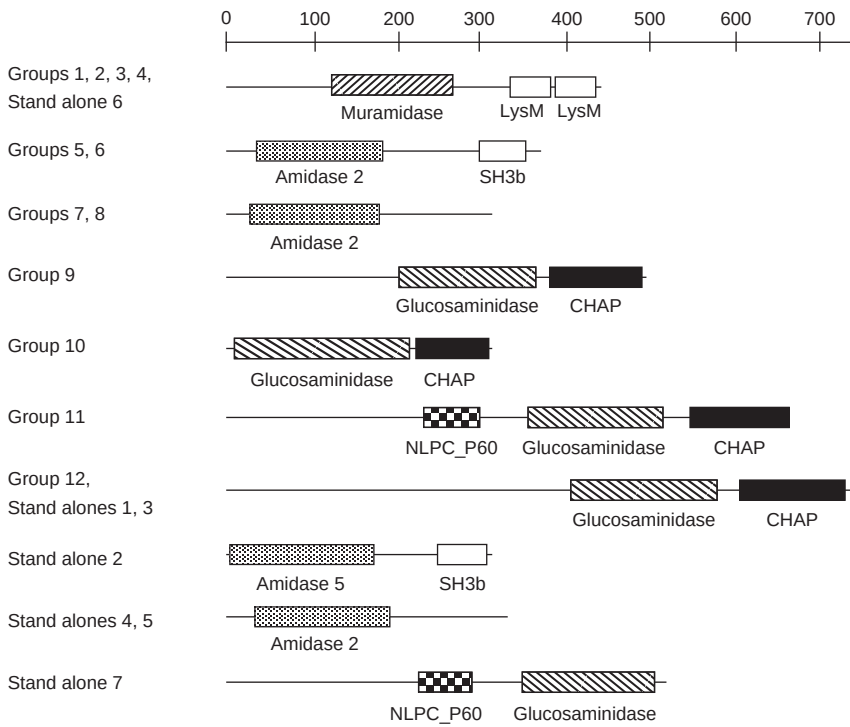

FIGURE 5 Enterococcal PG hydrolases. Groups are derived from homology clustering performed in BLAST, NCBI of the proteins described in Table III. Scale bar represents number of amino acids. Domains are defined more clearly in the PFAM database http://www.sanger.ac.uk/resources/databases/pfam.html. White boxes represent CBDs. LysM, (Bateman and Bycroft, 2000; Joris *et al.*, 1992); SH3b, bacterial Src homology 3 domain (Ponting *et al.*, 1999; Whisstock and Lesk, 1999); NLP_P60 (Anantharaman and Aravind, 2003).

4. Endolysins with multiple catalytic domains

Although it is well established that single domain endolysins can lyse the target pathogen (Sanz *et al.*, 1996), numerous endolysins 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 D-alanyl-L-alanyl endopeptidase catalytic activity on purified PG (Pritchard *et al.*, 2004), (3) the streptococcal λSa2 phage endolysin has 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

TABLE I Staphylococcal PG hydrolases

	AA	Accession #
Group 1		
Putative lysin [*Staphylococcus* phage K]	495	YP_024461
Endolysin [*Staphylococcus* phage 812]	494	ABL87139
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
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

TABLE I (*continued*)

	AA	Accession #
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
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
Group 9		
Autolysin (*S. aureus*)[3]	481	LYTA_STAAU

(*continued*)

TABLE I (*continued*)

	AA	Accession #
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
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

TABLE I (*continued*)

	AA	Accession #
Mannosyl-glycoprotein endo-β-*N*-acetylglucosaminidase [*S. aureus* subsp. *aureus* JH9]	632	YP_001246286
Mannosyl-glycoprotein endo-β-*N*-acetylglucosaminidase [*S. aureus* A8115]	632	ZP_05690673
Mannosyl-glycoprotein endo-β-*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-β-*N*-acetylglucosaminidase [*S. aureus* subsp. *aureus* D139]	632	ZP_06324913
Mannosyl-glycoprotein endo-β-*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
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-β-*N*-acetylglucosaminidase [*S. aureus* subsp. *aureus* 132]	624	ZP_06378620
Mannosyl-glycoprotein endo-β-*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

(*continued*)

TABLE I (*continued*)

	AA	Accession #
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-β-*N*-acetylglucosaminidase [*S. aureus* subsp. *aureus* JKD6009]	624	ZP_03566885
Hypothetical protein HMPREF0776_1895 [*S. aureus* subsp. *aureus* USA300_TCH959]	624	ZP_04865678
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-β-*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 [*S. 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
	463	YP_189215

TABLE I (*continued*)

	AA	Accession #
12 Prophage, amidase, putative [*S. epidermidis* RP62A]		
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-β-*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 $\geq$90%.
Exceptions: [1]89%; [2]87%; [3]89%; [4]89%; [5]88%; [6]88%; [7]89%; [8]87%; [9]86%; [10]84%.

TABLE II Streptococcal PG hydrolases

	AA	Accession #
Group 1a		
Cpl-1 [*S. pneumoniae*]	339	NP_044837.1
Cpl-9 [*S. pneumoniae*]	339	P19386.1
Group 1b		
PH10 lysin [*S. oralis*]	334	YP_002925184.1
Group 1c		
Cpl-7 [*S. pneumoniae*]	342	P19385.1
Group 2a		
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
Group 2b		
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

TABLE II (*continued*)

	AA	Accession #
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 2c		
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 2d		
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

(*continued*)

TABLE II (*continued*)

	AA	Accession #
Group 3a		
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 3b		
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 3c		
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 3d		
Phage-associated lysin [*S. equi* subsp. *equi* 4047]	404	YP_002745608.1
Phage amidase [*S. equi* subsp. *equi* 4047]	403	YP_002746965.1
Group 3e		
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

TABLE II (*continued*)

	AA	Accession #
Group 3f		
spyM18_1242 [*S. pyogenes* MGAS8232]	161	NP_607353.1
Group 3g		
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 5a		
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 5b		
Phage-associated lysin [*S. pyogenes* MGAS10394]	203	YP_060515.1
Group 6a		
Phage 9429.2 lysin [*S. pyogenes*]	373	YP_596581.1
Group 6b		
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* 2603 V/R]	239	NP_687631.1
Endolysin [*S. agalactiae* H36B]	248	ZP_00782522.1

(*continued*)

TABLE II (*continued*)

	AA	Accession #
Group 8a		
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 8b		
LambdaSa2 lysin [*S. dysgalactiae* subsp. *equisimilis* GGS_124]	449	YP_002997317.1
Group 8c		
LambdaSa2 lysin [*S. agalactiae* 2603 V/R]	468	NP_688827.1
Group 8d		
SMP lysin [*S. suis*]	481	YP_950557.1
Group 9a		
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-β-*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 9b		
Endo-β-*N*-acetylglucosaminidase [*S. pneumoniae* 70585]	702	YP_002740268.1
Endo-β-*N*-acetylglucosaminidase [*S. pneumoniae* G54]	702	YP_002037600.1
Endo-β-*N*-acetylglucosaminidase [*S. pneumoniae* Hungary19A-6]	702	YP_001694410.1
Endo-β-*N*-acetylglucosaminidase [*S. pneumoniae* P1031]	702	YP_002738134.1
Endo-β-*N*-acetylglucosaminidase [*S. pneumoniae* Taiwan19F-14]	702	YP_002742657.1
Endo-β-*N*-acetylglucosaminidase [*S. pneumoniae* BS397]	702	ZP_07350631.1
Group 9c		
Endo-β-*N*-acetylglucosaminidase [*S. pneumoniae* SP-BS293]	614	ZP_07345852.1

TABLE II (*continued*)

	AA	Accession #
Endo-β-*N*-acetylglucosaminidase [*S. pneumoniae*]	614	AAK19156.1
Endo-β-*N*-acetylglucosaminidase [*S. pneumoniae* CDC1087-00]	614	ZP_02710425.1
Endo-β-*N*-acetylglucosaminidase [*S. pneumoniae* INV104]	614	CBW36509.1
LytB [*Spneumoniae* AP200]	614	YP_003876588.1
Group 9d		
Endo-β-*N*-acetylglucosaminidase [*S. pneumoniae* CGSP14]	677	YP_001835658.1
Group 9e		
Endo-β-*N*-acetylglucosaminidase [*S. pneumoniae* CCRI 1974]	658	ZP_04525138.1
Endo-β-*N*-acetylglucosaminidase [*S. pneumoniae* CDC0288-04]	658	ZP_02715197.1
Endo-β-*N*-acetylglucosaminidase [*S. pneumoniae* CDC3059-06]	658	ZP_02718537.1
Endo-β-*N*-acetylglucosaminidase [*S. pneumoniae* JJA]	658	YP_002735981.1
Endo-β-*N*-acetylglucosaminidase [*S. pneumoniae* SP23-BS72]	658	ZP_01834875.1
Endo-β-*N*-acetylglucosaminidase [*S. pneumoniae* MLV-016]	658	ZP_02721563.1
Endo-β-*N*-acetylglucosaminidase [*S. pneumoniae* TIGR4]	658	NP_345446.1
Endo-β-*N*-acetylglucosaminidase [*S. pneumoniae* SP3-BS71]	658	ZP_01817975.1
Group 9f		
Endo-β-*N*-acetylglucosaminidase [*S. pneumoniae* INV200]	721	CBW34519.1
Endo-β-*N*-acetylglucosaminidase [*S. pneumoniae* R6]	721	NP_358461.1
Endo-β-*N*-acetylglucosaminidase [*S. pneumoniae* TCH8431/19A]	721	YP_003724965.1
Group 10		
Endo-β-*N*-acetylglucosaminidase [*S. mitis* ATCC6249]	750	ZP_07462509.1
Endo-β-*N*-acetylglucosaminidase [*S. sanguinis* ATCC49296]	750	ZP_07887886.1

(*continued*)

TABLE II (*continued*)

	AA	Accession #
Endo-β-*N*-acetylglucosaminidase [*Streptococcus* sp. oral taxon str. 73H25AP]	750	ZP_07458768.1
Group 11a		
Lysozyme [*S. mitis* NCTC 12261]	525	ZP_07644807.1
LytC Cpb13 [*S. mitis* B6]	536	YP_003446665.1
Group 11b		
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 11c		
Lysozyme [*S. pneumoniae* SP3-BS71]	270	ZP_01818179.1
Group 11d		
1,4-β-*N*-Acetylmuramidase [*S. pneumoniae* CDC1873-00]	490	ZP_02708500.1
1,4-β-*N*-Acetylmuramidase [*S. pneumoniae* P1031]	490	YP_002738710.1
1,4-β-*N*-Acetylmuramidase [*S. pneumoniae* SP11-BS70]	490	ZP_01824964.1
1,4-β-*N*-Acetylmuramidase [*S. pneumoniae* SP9-BS68]	490	ZP_01822918.1
1,4-β-*N*-Acetylmuramidase [*S. pneumoniae* 70585]	490	YP_002740840.1
1,4-β-*N*-Acetylmuramidase [*S. pneumoniae* CDC1087-00]	490	ZP_02711346.1
1,4-β-*N*-Acetylmuramidase [*S. pneumoniae* TCH8431/19A]	501	YP_003725251.1
1,4-β-*N*-Acetylmuramidase [*S. pneumoniae* R6]	501	NP_359024.1
1,4-β-*N*-Acetylmuramidase [*S. pneumoniae*]	492	AAK19157.1
ATP-dependent protease [*S. pneumoniae* SP6-BS73]	490	ZP_01820060.1
Endo-β-*N*-acetylglucosaminidase [*S. pneumoniae* G54]	490	YP_002038205.1
Lysozyme [*S. pneumoniae* Taiwan 19 F-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

TABLE II (*continued*)

	AA	Accession #
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 19 F]	490	ZP_06964203.1
SpneT 0200379 [*S. pneumoniae* TIGR4]	490	ZP_01409152.1
Group 11e		
1,4-β-*N*-Acetylmuramidase [*S. pneumoniae* SP14-BS69]	311	ZP_01828088.1
Group 11f		
Lysozyme [*S. pneumoniae* SP19-BS75]	227	ZP_01833670.1
Group 12a		
Pal [*S. pneumoniae* phage DP-1]	296	O03979.1
Group 12b		
gp56 [*Streptococcus* phage SM1]	295	NP_862895.1
Group 13a		
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 13b		
Putative phage PH15 endolysin [*S. gordonii*]	283	YP_001974380.1
Group 13c		
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 13d		
Sfi11 lysin [*S. thermophilus*]	288	NP_056699.1
Sfi18 lysin [*S. thermophilus*]	288	AAF63073.1

(*continued*)

TABLE II (*continued*)

	AA	Accession #
Sfi19 lysin [*S. thermophilus*]	288	NP_049942.1
Sfi21 lysin [*S. thermophilus*]	288	NP_049985.1
Group 13e		
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 gp19 [*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.

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 phenomenon 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 *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 to have a very active N-terminal D-alanyl-glycyl endopeptidase domain via deletion analysis (Donovan *et al.*, 2006c; Sass and Bierbaum, 2007) and a nearly silent *N*-acetylmuramoyl-L-

TABLE III Enterococcal PG hydrolases

	AA	Accession #
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

(*continued*)

TABLE III (*continued*)

	AA	Accession #
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

TABLE III (*continued*)

	AA	Accession #
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

(*continued*)

TABLE III (*continued*)

	AA	Accession #
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 $\geq$90%. Exception: [1]89%.

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 (D. M. Donovan, 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. What is needed are a series of experiments that test the effect of a mutant endolysin gene, with either the active or the 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 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, what follows 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, nonviable 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 are considerations for whether (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 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

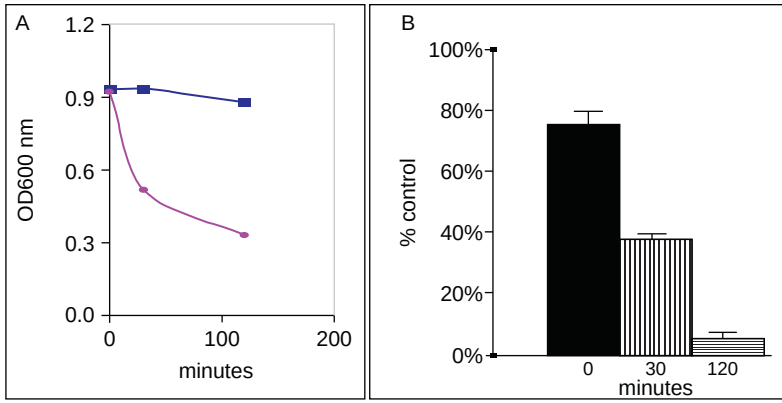

FIGURE 6 A reduction in turbidity equates to reduced bacterial viability. (A) Twenty-five micrograms of Φ11 endolysin [construct Φ11-194 (Donovan *et al*., 2006)] protein (circles) and *S. aureus* cells alone (squares) were monitored for 120 min in a turbidity reduction assay. (B) Treated (Φ11-194) and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

and bacterial PG hydrolases reportedly range from 10^2 to 10^8 ''units'' per milligram 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 sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis 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 hr in a buffer compatible with the lytic enzyme to remove the SDS. Appearance of a cleared region in the opaque gel indicates that 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 2 × 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 37 °C. After 20 hr, 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 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 (D. M. Donovan, unpublished data).

Plate lysis (spot on lawn): A log growth-phase culture of 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 temperature. Ten microliter-aliquots of known concentration(s) of the PG hydrolase are spotted onto the lawn and allowed to air dry (≈10 min) at room temperature. Plates are incubated at optimal growth temperature, and plates are 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., isopropyl-β-D-thiogalactoside) are created from original agar plates containing transformant colonies. The replica plates are incubated at 37 °C for up to 6 hr 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 are 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 hr), 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, 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 and Kokai-Kun (2005), acknowledging that bacteria express different surface factors in liquid media than on solid media (culture media can affect 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, as the MIC assay measures growth inhibition while PG hydrolases probably kill the initial inocula rapidly.

F. Cell wall-binding domains of Gram-positive endolysins

Numerous domains have been assigned CBD status (see Figs. 3–5). 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 domain of the pneumococcal amidase autolysin, which requires choline or ethanolamine to achieve full activation (Garcia *et al.*, 1990). 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 its homologue ALE-1 that is 80% identical in both lytic domains 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). It has been reported 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, CBD recognition of an epitope is analogous to recognition of a cell surface receptor by a phage tail fiber. In fact, some evidence shows 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 *Bacillus 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, 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* (D. C. Nelson, 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 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 a century, particularly those endolysins from the T-even phage that infect Gram-negative hosts. However, it has only been since 2001 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 IV shows a complete list to date of all *in vivo* therapeutic trials that utilize bacteriophage-encoded endolysins, which are summarized here.

Nelson *et al.* (2001) were the first to use a purified phage endolysin in an *in vivo* model. It was found that oral administration of an endolysin (250 U) from the streptococcal C1 bacteriophage provided protection from upper respiratory colonization in mice challenged with 10^7 *Streptococcus*

TABLE IV Summary of *in vivo* studies with phage endolysins as antimicrobials.

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 pneumonia	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	CHAPk	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

pyogenes (i.e., group A streptococci) (28.5% infected for endolysin treatment vs 70.5% infected for phosphate-buffered treatment). Furthermore, when 500 U of this streptococcal endolysin, named PlyC in a later publication (Nelson *et al.*, 2006), was administered orally to nine heavily colonized mice, no detectable streptococci were observed via oral swabs 2 hr 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 streptococci) 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. PAL (100 U/ml) was shown to cause a ≈4 log drop in viability in 30 s of 15 different *Streptococcus pneumoniae* serotypes representing multidrug-resistant isolates and those that contain a heavy polysaccharide capsule (Loeffler *et al.*, 2001). In a mouse model of nasopharyngeal carriage, 1400 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 both in 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 pneumococci in a rat endocarditis model (Entenza *et al.*, 2005) [although biofilms were not specifically described, it is widely accepted that biofilms play a major role in endocarditis (Simões, 2011)]. Infusion of 250 mg/kg was able to sterilize 10^5 colony-forming unit (CFU)/ml pneumococci in blood within 30 min and reduce bacterial titers on heart valve vegetations by >4 log CFU/g in 2 hr. 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 reduce secondary 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. Fifty units 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 (Schuch *et al.*, 2002). Furthermore, of the mice that died, fatal infection took between 6 and 14 hr to develop, whereas all non-PlyG-treated mice died

within 5 hr. 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 *Bacillus* endolysins make them amenable to therapeutic treatment and diagnostics of *B. anthracis*.

The prevalence of methicillin-resistant *S. 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 lysed all tested staphylococcal strains rapidly, 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 min postinfection. At 60 min postinfection, 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 CBD of the ΦNM3 phage endolysin (Daniel *et al.*, 2010). Like MV-L, this enzyme displayed potent bacteriolytic properties against multidrug-resistant staphylococci *in vitro*. In a mouse MRSA colonization model, 2-log reductions in viability were observed 1 hr following a single treatment of 960 μg ClyS. Similarly, a single dose (1 mg) of ClyS provided protection when administered 3 hr post-staphylococcal challenge in an intraperitoneal septicemia model. Notably, ClyS showed synergy *in vivo* with oxacillin at doses that were not protective individually against MRSA in this 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, caused a 2 log drop in nasal colonization of mice 1 hr post-treatment (Fenton *et al.*, 2010a,b).

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, 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). The transgenic cows produced lysostaphin at concentrations ranging from 0.9 to 14 μg/ml in their milk. Protection appeared to be dose dependent, with a minimum concentration of 3 μg/ml in milk required for complete protection.

B. Immune responses

Due to the proteinaceous nature of PG hydrolases and their potential use as human and animal therapeutics, potential adverse immune responses must be considered, 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 *B. anthracis* (PlyG), *S. pyogenes* (PlyC), or *S. pneumoniae* (Pal). When high titers of these antibodies were mixed *in vitro* with endolysins, killing of the target microbe was slowed, but not stopped (Fischetti, 2005; Loeffler *et al.*, 2003). In another study, Cpl-1, a pneumococcal endolysin, was injected intravenously (IV) three times per week into mice for 4 weeks, resulting in positive IgG antibodies against Cpl-1 in five of six 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 hr. Bacteremic titers were reduced within 1 min 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 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 IV route produced serum antibodies to lysostaphin that resulted in an eightfold 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, when used alone, lead to selection for resistant strains, not just in the target pathogen, but also in coresident commensal bacteria exposed to the drug. The acquisition of antibiotic resistance is often accomplished by the transfer of DNA sequences from a resistant strain to a susceptible strain (Johnsborg and Håvarstein, 2009). This transfer is not necessarily species or genus limited and can lead to commensal bacteria that are both antibiotic resistant and 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 distributed further 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. 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 (2005) is that the bacterial host and phage have coevolved such that the phage might have evolved endolysins to target immutable bonds in order to ensure its survival and release from the host. Thus, resistance to phage endolysins is expected to be a very rare event.

Despite the lack of observed resistance in 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

and 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 reviews, see Davis and Weiser (2011) and Vollmer (2008)].

Similarly, at least two genes 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 subinhibitory 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 fivefold less virulent in a rodent kidney infection model, and were treated easily with β-lactam antibiotics (Kusuma *et al.*, 2007).

Grundling *et al.* (2006) identified the *lyrA* gene (lysostaphin resistance A) that, when mutated by a transposon gene insertion, reduced staphylococcal susceptibility to lysostaphin. 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, 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); 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 MRSA. This combination is uniquely promising in that when lysostaphin-resistant staphylococci are generated by modifying the pentaglycine bridge of the PG; 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 Pal 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.*, 2010a). Nisin was also shown to be synergistic with lysozyme against lactic acid bacteria (Chun and Hancock, 2000). Finally, ClyS, a fusion lysin described earlier, 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 multifaceted 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 have an extensive extracellular matrix. The National Institutes of Health (NIH) estimate 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 that 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; Høiby *et al.*, 2010; Keren *et al.*, 2004; Lewis, 2001).

Little work has been done to specifically test phage endolysins for their antibiofilm 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). 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 antibiofilm 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; endolysins effective against *B. 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 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 noncorrosive (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, endolysins may not be as susceptible to dilution factors as 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 min 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 1000 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 min. Based on these findings, the authors performed a standard battery of tests

approved by the Association of Official Analytical Chemists, including the use dilution method for testing disinfectants and 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 the equipment of various porosities found commonly in horse stables. Finally, PlyC was shown to retain effectiveness when tested in the presence of nonionic detergents, hard water, and organic material.

G. Food safety

The use of phage and phage products for use in food safety has been reviewed (Hagens and Loessner, 2010; Hermoso *et al.*, 2007; O'Flaherty *et al.*, 2009). ListShield and Ecoshield from Intralytix and LISTEX^{TM} from MICREOS Food Safety are phage preparations designed to protect food from *L. monocytogenes* or *Escherichia coli.* One regulatory distinction between phages and endolysins is that phages 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 (Callewaert *et al.*, 2010; Garcia *et al.*, 2010b; 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 U.S. Food and Drug Administration for the use of *Listeria* bacteriophage on sliced meat products (http://edocket.access.gpo.gov/2006/pdf/E6-13621.pdf).

Peptidoglycan hydrolases are effective antimicrobials when introduced into foodstuffs 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 transgenic milk (Maga *et al.*, 2006a,b). 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: as a final stage to protect food products from food pathogens or a method to protect crop production from plant pests, and 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 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).

Nontransgenic 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 shown to be synergistic with nisin, a lactococcal bacteriocin that has achieved generally recognized as safe status (Garcia *et al.*, 2010a). 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 (Simmons *et al.*, 2010; Zimmer *et al.*, 2002). 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 foodstuffs 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,b; Lopez *et al.*, 1997). In a reverse approach, a clostridial CBD was fused C-terminally to a catalytic domain of the pneumococcal autolysin LytA,

increasing its activity against clostridial cell walls considerably (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 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 improved the solubility of the fusion over the parental hydrolases greatly, allowing purification and experiments to demonstrate strong antimicrobial activity toward *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 the 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 earlier is reported to be effective at curing murine topical infections of *S. aureus* (Pastagia *et al.*, 2011) and is effective in combination with classical antibiotics at eradicating multidrug-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 study examined the effect of an N- or C-terminal His tag on lysostaphin with the resultant activities being 80 and 20% of the nontagged version, respectively (Becker *et al.*, 2011). That same publication also looked at microdeletions (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, 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 and Kokai-Kun (2005); that finding was confirmed with turbidity reduction and plate lysis assays where N-terminal microdeletions 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 *et al.* (2004) altered conserved amino acids in the streptococcal B30 endolysin CHAP and lysozyme domains, which resulted in a 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 *B. anthracis* phage lysin PlyG were essential in defining the binding domain and active site residues (Kikkawa *et al.*, 2007, 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- to 10-fold reduction in lytic activity compared to wild-type lysostaphin (Kerr *et al.*, 2001). By separating the two altered residues on separate constructs, it was shown 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, for example, the staphylococcal LysK (Horgan *et al.*, 2009). One such hyperactive 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 that 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 a physiological salt concentration, which again may be explained by a loss of surface mobility (Schmelcher *et al.*, 2011).

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.* (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 outlined earlier, 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. 3–5). Dual domain endolysins are predicted to be more refractory to resistant strain development (Fischetti, 2005). The Donovan laboratory 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.

The authors have created several triple-lytic-domain anti-staphylococcal fusion constructs using the synergistic enzymes LysK and lysostaphin. Lysostaphin and LysK collectively harbor three cleavage domains that cleave at unique sites (described earlier). 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 *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 Portillo and Finlay, 1995). Alternative drug treatment systems for the delivery of antimicrobials to intracellular pathogens have been described (Imbuluzqueta *et al.*, 2010).

One 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, whereas others require pinocytotic uptake (Duchardt *et al.*, 2007; Joliot and Prochiantz, 2004). There are reports of noncharged 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, which is highly effective at excluding large molecules and is 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 exposed directly 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 m*M* EDTA, used in combination with 50 μg/ml of the *Pseudomonas* endolysin EL188, decreased viable *P. aeruginosa* cells by 3 or 4 logs in 30 min 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 fused to them genetically 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. Nonenzymatic domains and recent successes

Some PG hydrolases and endolysins can kill pathogens via a mechanism completely separate from their ability to cleave the PG enzymatically. For example, the 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 showed 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 referred to collectively as host-defense peptides (Sahl and Bierbaum, 2008).

Similar to the T4 lysozyme, several additional endolysins have been identified that contain amphipathic or highly cationic regions in addition to their catalytic domains. Preliminary studies suggest that 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. baumannii* phage, was found to degrade isolated cell walls of *A. baumannii* 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. baumannii*, *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 catalytically inactive mutants 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 successes have inspired renewed interest in the use of endolysins against Gram-negative bacteria, an idea once considered a nonstarter. 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 with the goal to improve endolysin-based therapy to Gram-negative pathogens will expand greatly in the coming years.

C. High-pressure treatment

In another approach, the use of high hydrostatic pressure (HHP) can dramatically increase the 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, 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, 2001; Nakimbugwe *et al.*, 2006).

Nakimbugwe *et al.* (2006) tested HHP in conjunction with six individual PG hydrolases, including phage endolysins from λ and T4, on 10 different bacterial strains (five each of Gram negative and positive). Both phage endolysins were active on four out of five of the Gram-negative bacteria and *Bacillus subtilis*, although 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 with lytic transglycosylase activity, 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

Multidrug-resistant superbugs have ''raised the bar'' in establishing a higher set of requirements for new antimicrobials. New antimicrobial agents should ideally eradicate multidrug-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 unrelated 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 nondividing or slowly growing cells, for example, 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 PTDs is nontrivial in light of the toxic levels required for most drugs to

eradicate pathogens residing intracellularly. Similarly, PG hydrolases are synergistic with many classes of classical antimicrobials, thus potentially extending the clinical half-life of overused 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 that 80% and Centers for Disease Control and Prevention estimating that 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. The authors believe that PG hydrolases are an ideal candidate class of novel antimicrobials with which to address these inevitable concerns.

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REFERENCES

Amorena, B., Gracia, E., Monzon, M., Leiva, J., Oteiza, C., Perez, M., Alabart, J. L., and Hernandez-Yago, J. (1999). Antibiotic susceptibility assay for *Staphylococcus aureus* in biofilms developed *in vitro*. *J. Antimicrob. Chemother.* **44:**43–55.

Anantharaman, V., and Aravind, L. (2003). Evolutionary history, structural features and biochemical diversity of the NlpC/P60 superfamily of enzymes. *Genome Biol.* **4:**R11.

Baba, T., and Schneewind, O. (1996). Target cell specificity of a bacteriocin molecule: a C-terminal signal directs lysostaphin to the cell wall of *Staphylococcus aureus*. *EMBO J.* **15:**4789–4797.

Baker, J. R., Liu, C., Dong, S., and Pritchard, D. G. (2006). Endopeptidase and glycosidase activities of the bacteriophage B30 lysin. *Appl. Environ. Microbiol.* **72:**6825–6828.

Bateman, A., and Bycroft, M. (2000). The structure of a LysM domain from *E. coli* membrane-bound lytic murein transglycosylase D (MltD). *J. Mol. Biol.* **299:**1113–1119.

Bateman, A., and Rawlings, N. D. (2003). The CHAP domain: A large family of amidases including GSP amidase and peptidoglycan hydrolases. *Trends Biochem. Sci.* **28:** 234–237.

Becker, S. C., Dong, S., Baker, J. R., Foster-Frey, J., Pritchard, D. G., and Donovan, D. M. (2009a). LysK CHAP endopeptidase domain is required for lysis of live staphylococcal cells. *FEMS Microbiol. Lett.* **294:**52–60.

Becker, S. C., Foster-Frey, J., and Donovan, D. M. (2008). The phage K lytic enzyme LysK and lysostaphin act synergistically to kill MRSA. *FEMS Microbiol. Lett.* **287:**185–191.

Becker, S. C., Foster-Frey, J., Powell, A., Kerr, D., and Donovan, D. M. (2011). Lysostaphin: Molecular changes that preserve staphylolytic activity. *In* ''2010 International Conference on Antimicrobial Research'' (A. Mendez-Vilas, ed.), pp. 18–22. World Scientific Publishing Co., Singapore.

Becker, S. C., Foster-Frey, J., Stodola, A. J., Anacker, D., and Donovan, D. M. (2009b). Differentially conserved staphylococcal SH3b_5 cell wall binding domains confer increased staphylolytic and streptolytic activity to a streptococcal prophage endolysin domain. *Gene* **443:**32–41.

Boothe, J., Nykiforuk, C., Shen, Y., Zaplachinski, S., Szarka, S., Kuhlman, P., Murray, E., Morck, D., and Moloney, M. M. (2010). Seed-based expression systems for plant molecular farming. *Plant Biotechnol. J.* **8:**588–606.

Borysowski, J., and Gorski, A. (2010). Fusion to cell-penetrating peptides will enable lytic enzymes to kill intracellular bacteria. *Med. Hypotheses* **74:**164–166.

Bramley, A. J., and Foster, R. (1990). Effects of lysostaphin on *Staphylococcus aureus* infections of the mouse mammary gland. *Res. Vet. Sci.* **49:**120–121.

Briers, Y., Cornelissen, A., Aertsen, A., Hertveldt, K., Michiels, C. W., Volckaert, G., and Lavigne, R. (2008). Analysis of outer membrane permeability of *Pseudomonas aeruginosa* and bactericidal activity of endolysins KZ144 and EL188 under high hydrostatic pressure. *FEMS Microbiol. Lett.* **280:**113–119.

Briers, Y., Schmelcher, M., Loessner, M. J., Hendrix, J., Engelborghs, Y., Volckaert, G., and Lavigne, R. (2009). The high-affinity peptidoglycan binding domain of *Pseudomonas* phage endolysin KZ144. *Biochem. Biophys. Res. Commun.* **383:**187–191.

Briers, Y., Volckaert, G., Cornelissen, A., Lagaert, S., Michiels, C. W., Hertveldt, K., and Lavigne, R. (2007). Muralytic activity and modular structure of the endolysins of *Pseudomonas aeruginosa* bacteriophages phiKZ and EL. *Mol. Microbiol.* **65:**1334–1344.

Briers, Y., Walmagh, M., and Lavigne, R. (2011). Use of bacteriophage endolysin EL188 and outer membrane permeabilizers against *Pseudomonas aeruginosa*. *J. Appl. Microbiol.* **110** (3)**:**778–785.

Bustamante, N., Campillo, N. E., Garcia, E., Gallego, C., Pera, B., Diakun, G. P., Saiz, J. L., Garcia, P., Diaz, J. F., and Menendez, M. (2010). Cpl-7, a lysozyme encoded by a pneumococcal bacteriophage with a novel cell wall-binding motif. *J Biol. Chem.* **285:**33184–33196.

Callewaert, L., Walmagh, M., Michiels, C. W., and Lavigne, R. (2010). Food applications of bacterial cell wall hydrolases. *Curr. Opin. Biotechnol.* **22:**164–171.

Cheng, Q., and Fischetti, V. A. (2007). Mutagenesis of a bacteriophage lytic enzyme PlyGBS significantly increases its antibacterial activity against group B streptococci. *Appl. Microbiol. Biotechnol.* **74:**1284–1291.

Cheng, Q., Nelson, D., Zhu, S., and Fischetti, V. A. (2005). Removal of group B streptococci colonizing the vagina and oropharynx of mice with a bacteriophage lytic enzyme. *Antimicrob. Agents Chemother.* **49:**111–117.

Chun, W., and Hancock, R. E. (2000). Action of lysozyme and nisin mixtures against lactic acid bacteria. *Int. J. Food Microbiol.* **60:**25–32.

Climo, M. W., Ehlert, K., and Archer, G. L. (2001). Mechanism and suppression of lysostaphin resistance in oxacillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **45:**1431–1437.

Climo, M. W., Patron, R. L., Goldstein, B. P., and Archer, G. L. (1998). Lysostaphin treatment of experimental methicillin-resistant *Staphylococcus aureus* aortic valve endocarditis. *Antimicrob. Agents Chemother.* **42:**1355–1360.

Croux, C., Ronda, C., Lopez, R., and Garcia, J. L. (1993a). Interchange of functional domains switches enzyme specificity: Construction of a chimeric pneumococcal-clostridial cell wall lytic enzyme. *Mol. Microbiol.* **9:**1019–1025.

Croux, C., Ronda, C., Lopez, R., and Garcia, J. L. (1993b). Role of the C-terminal domain of the lysozyme of *Clostridium acetobutylicum* ATCC 824 in a chimeric pneumococcal-clostridial cell wall lytic enzyme. *FEBS Lett.* **336:**111–114.

Cui, F., Li, G., Huang, J., Zhang, J., Lu, M., Lu, W., Huan, J., and Huang, Q. (2011). Development of chitosan-collagen hydrogel incorporated with lysostaphin (CCHL) burn dressing with anti-methicillin-resistant *Staphylococcus aureus* and promotion wound healing properties. *Drug Deliv.* **18:**173–180.

Dajcs, J. J., Austin, M. S., Sloop, G. D., Moreau, J. M., Hume, E. B., Thompson, H. W., McAleese, F. M., Foster, T. J., and O'Callaghan, R. J. (2002). Corneal pathogenesis of *Staphylococcus aureus* strain Newman. *Invest Ophthalmol. Vis. Sci.* **43:**1109–1115.

Dajcs, J. J., Hume, E. B., Moreau, J. M., Caballero, A. R., Cannon, B. M., and O'Callaghan, R. J. (2000). Lysostaphin treatment of methicillin-resistant *Staphylococcus aureus* keratitis in the rabbit(1). *Am. J. Ophthalmol.* **130:**544.

Dajcs, J. J., Thibodeaux, B. A., Hume, E. B., Zheng, X., Sloop, G. D., and O'Callaghan, R. J. (2001). Lysostaphin is effective in treating methicillin-resistant *Staphylococcus aureus* endophthalmitis in the rabbit. *Curr. Eye Res.* **22:**451–457.

Daniel, A., Euler, C., Collin, M., Chahales, P., Gorelick, K. J., and Fischetti, V. A. (2010). Synergism between a novel chimeric lysin and oxacillin protects against infection by methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **54:**1603–1612.

Davis, K. M., and Weiser, J. N. (2011). Modifications to the peptidoglycan backbone help bacteria to establish infection. *Infect. Immun.* **79:**562–570.

DeHart, H. P., Heath, H. E., Heath, L. S., LeBlanc, P. A., and Sloan, G. L. (1995). The lysostaphin endopeptidase resistance gene (epr) specifies modification of peptidoglycan cross bridges in *Staphylococcus simulans* and *Staphylococcus aureus*. *Appl. Environ. Microbiol.* **61:**1475–1479.

Delbruck, M. (1940). The growth of bacteriophage and lysis of the host. *J. Gen. Physiol* **23:**643–660.

Desbois, A. P., Lang, S., Gemmell, C. G., and Coote, P. J. (2010). Surface disinfection properties of the combination of an antimicrobial peptide, ranalexin, with an endopeptidase, lysostaphin, against methicillin-resistant *Staphylococcus aureus* (MRSA). *J. Appl. Microbiol.* **108:**723–730.

Deutsch, S. M., Guezenec, S., Piot, M., Foster, S., and Lortal, S. (2004). Mur-LH, the broad-spectrum endolysin of *Lactobacillus helveticus* temperate bacteriophage phi-0303. *Appl. Environ. Microbiol.* **70:**96–103.

Dhalluin, A., Bourgeois, I., Pestel-Caron, M., Camiade, E., Raux, G., Courtin, P., Chapot-Chartier, M. P., and Pons, J. L. (2005). Acd, a peptidoglycan hydrolase of *Clostridium difficile* with N-acetylglucosaminidase activity. *Microbiology* **151:**2343–2351.

Diaz, E., Lopez, R., and Garcia, J. L. (1991). Chimeric pneumococcal cell wall lytic enzymes reveal important physiological and evolutionary traits. *J. Biol. Chem.* **266:**5464–5471.

Diaz, E., Lopez, R., and Garcia, J. L. (1990). Chimeric phage-bacterial enzymes: A clue to the modular evolution of genes. *Proc. Natl. Acad. Sci. USA* **87:**8125–8129.

Djurkovic, S., Loeffler, J. M., and Fischetti, V. A. (2005). Synergistic killing of *Streptococcus pneumoniae* with the bacteriophage lytic enzyme Cpl-1 and penicillin or gentamicin depends on the level of penicillin resistance. *Antimicrob. Agents Chemother.* **49:**1225–1228.

Donlan, R. M. (2008). Biofilms on central venous catheters: Is eradication possible? *Curr. Top. Microbiol. Immunol.* **322:**133–161.

Donovan, D. M., and Foster-Frey, J. (2008). LambdaSa2 prophage endolysin requires Cpl-7-binding domains and amidase-5 domain for antimicrobial lysis of streptococci. *FEMS Microbiol. Lett.* **287:**22–33.

Donovan, D. M., Becker, S. C., Dong, S., Baker, J. R., Foster-Frey, J., and Pritchard, D. G. (2009). Peptidoglycan hydrolase enzyme fusions for treating multi-drug resistant pathogens. *Biotech. Intl.* **21:**6–10.

Donovan, D. M., Dong, S., Garrett, W., Rousseau, G. M., Moineau, S., and Pritchard, D. G. (2006a). Peptidoglycan hydrolase fusions maintain their parental specificities. *Appl. Environ. Microbiol.* **72:**2988–2996.

Donovan, D. M., Foster-Frey, J., Dong, S., Rousseau, G. M., Moineau, S., and Pritchard, D. G. (2006b). The cell lysis activity of the *Streptococcus agalactiae* bacteriophage B30 endolysin relies on the cysteine, histidine-dependent amidohydrolase/peptidase domain. *Appl. Environ. Microbiol.* **72:**5108–5112.

Donovan, D. M., Lardeo, M., and Foster-Frey, J. (2006c). Lysis of staphylococcal mastitis pathogens by bacteriophage phi11 endolysin. *FEMS Microbiol. Lett.* **265:**133–139.

Duchardt, F., Fotin-Mleczek, M., Schwarz, H., Fischer, R., and Brock, R. (2007). A comprehensive model for the cellular uptake of cationic cell-penetrating peptides. *Traffic* **8:**848–866.

During, K., Porsch, P., Fladung, M., and Lorz, H. (1993). Transgenic potato plants resistant to the phytopathogenic bacterium *Erwinia carotovora*. *Plant J.* **3:**587–598.

Dziarski, R., and Gupta, D. (2006). The peptidoglycan recognition proteins (PGRPs). *Genome Biol.* **7:**232.

Emori, T. G., and Gaynes, R. P. (1993). An overview of nosocomial infections, including the role of the microbiology laboratory. *Clin. Microbiol. Rev.* **6:**428–442.

Entenza, J. M., Loeffler, J. M., Grandgirard, D., Fischetti, V. A., and Moreillon, P. (2005). Therapeutic effects of bacteriophage Cpl-1 lysin against *Streptococcus pneumoniae* endocarditis in rats. *Antimicrob. Agents Chemother.* **49:**4789–4792.

Fenton, M., Casey, P. G., Hill, C., Gahan, C. G., Ross, R. P., McAuliffe, O., O'Mahony, J., Maher, F., and Coffey, A. (2010a). The truncated phage lysin CHAP(k) eliminates *Staphylococcus aureus* in the nares of mice. *Bioeng. Bugs.* **1:**404–407.

Fenton, M., Ross, P., McAuliffe, O., O'Mahony, J., and Coffey, A. (2010b). Recombinant bacteriophage lysins as antibacterials. *Bioeng. Bugs.* **1:**9–16.

Filatova, L. Y., Becker, S. C., Donovan, D. M., Gladilin, A. K., and Klyachko, N. L. (2010). LysK, the enzyme lysing *Staphylococcus aureus* cells: Specific kinetic features and approaches towards stabilization. *Biochimie* **92:**507–513.

Firczuk, M., and Bochtler, M. (2007). Folds and activities of peptidoglycan amidases. *FEMS Microbiol. Rev.* **31:**676–691.

Firczuk, M., Mucha, A., and Bochtler, M. (2005). Crystal structures of active LytM. *J. Mol. Biol.* **354:**578–590.

Fischetti, V. A. (2005). Bacteriophage lytic enzymes: Novel anti-infectives. *Trends Microbiol.* **13:**491–496.

Fischetti, V. A., Nelson, D., and Schuch, R. (2006). Reinventing phage therapy: Are the parts greater than the sum? *Nat. Biotechnol.* **24:**1508–1511.

Fischetti, V. A., Zabriskie, J. B., and Gotschlich, E. C. (1972). Physical, chemical, and biological properties of type 6 M-protein extracted with purified streptococcal phage-associated lysin. *In* ''Fifth International Symposium on *Streptococcus pyogenes*'' (M. J. Haverkorn, ed.), p. 26. Excerpta Medica, Amsterdam.

Foley, S., Bruttin, A., and Brussow, H. (2000). Widespread distribution of a group I intron and its three deletion derivatives in the lysin gene of *Streptococcus thermophilus* bacteriophages. *J. Virol.* **74:**611–618.

Fujinami, Y., Hirai, Y., Sakai, I., Yoshino, M., and Yasuda, J. (2007). Sensitive detection of *Bacillus anthracis* using a binding protein originating from gamma-phage. *Microbiol. Immunol.* **51:**163–169.

Fujiwara, T., Aoki, S., Komatsuzawa, H., Nishida, T., Ohara, M., Suginaka, H., and Sugai, M. (2005). Mutation analysis of the histidine residues in the glycylglycine endopeptidase ALE-1. *J. Bacteriol.* **187:**480–487.

Fukushima, T., Kitajima, T., Yamaguchi, H., Ouyang, Q., Furuhata, K., Yamamoto, H., Shida, T., and Sekiguchi, J. (2008). Identification and characterization of novel cell wall hydrolase CwlT: A two-domain autolysin exhibiting N-acetylmuramidase and DL-endopeptidase activities. *J. Biol. Chem.* **283:**11117–11125.

Fukushima, T., Yao, Y., Kitajima, T., Yamamoto, H., and Sekiguchi, J. (2007). Characterization of new L, D-endopeptidase gene product CwlK (previous YcdD) that hydrolyzes peptidoglycan in *Bacillus subtilis*. *Mol. Genet. Genomics* **278:**371–383.

Fux, C. A., Stooodley, P., Hall-Stoodley, L., and Costerton, J. W. (2003). Bacterial biofilms: A diagnostic and therapeutic challenge. *Expert Rev. Anti. Infect. Ther.* **1:**667–683.

Gaeng, S., Scherer, S., Neve, H., and Loessner, M. J. (2000). Gene cloning and expression and secretion of *Listeria monocytogenes* bacteriophage-lytic enzymes in *Lactococcus lactis*. *Appl. Environ. Microbiol.* **66:**2951–2958.

Garcia, J. L., Garcia, E., Arraras, A., Garcia, P., Ronda, C., and Lopez, R. (1987). Cloning, purification, and biochemical characterization of the pneumococcal bacteriophage Cp-1 lysin. *J. Virol.* **61:**2573–2580.

Garcia, P., Garcia, J. L., Garcia, E., Sanchez-Puelles, J. M., and Lopez, R. (1990). Modular organization of the lytic enzymes of *Streptococcus pneumoniae* and its bacteriophages. *Gene* **86:**81–88.

Garcia, P., Martinez, B., Rodriguez, L., and Rodriguez, A. (2010a). Synergy between the phage endolysin LysH5 and nisin to kill *Staphylococcus aureus* in pasteurized milk. *Int. J. Food Microbiol.* **141:**151–155.

Garcia, P., Rodriguez, L., Rodriguez, A., and Martinez, B. (2010b). Food biopreservation: Promising strategies using bacteriocins, bacteriophages and endolysins. *Trends Food Sci. Technol.* **21:**373–382.

Garcia-del Portillo, F., and Finlay, B. B. (1995). The varied lifestyles of intracellular pathogens within eukaryotic vacuolar compartments. *Trends Microbiol.* **3:**373–380.

Graham, S., and Coote, P. J. (2007). Potent, synergistic inhibition of *Staphylococcus aureus* upon exposure to a combination of the endopeptidase lysostaphin and the cationic peptide ranalexin. *J. Antimicrob. Chemother.* **59:**759–762.

Grandgirard, D., Loeffler, J. M., Fischetti, V. A., and Leib, S. L. (2008). Phage lytic enzyme Cpl-1 for antibacterial therapy in experimental pneumococcal meningitis. *J. Infect. Dis.* **197:**1519–1522.

Grundling, A., Missiakas, D. M., and Schneewind, O. (2006). *Staphylococcus aureus* mutants with increased lysostaphin resistance. *J. Bacteriol.* **188:**6286–6297.

Grundling, A., and Schneewind, O. (2006). Cross-linked peptidoglycan mediates lysostaphin binding to the cell wall envelope of *Staphylococcus aureus*. *J. Bacteriol.* **188:**2463–2472.

Gu, J., Xu, W., Lei, L., Huang, J., Feng, X., Sun, C., Du, C., Zuo, J., Li, Y., Du, T., Li, L., and Han, W. (2011). LysGH15, a novel bacteriophage lysin, protects a murine bacteremia model efficiently against lethal methicillin-resistant *Staphylococcus aureus* infection. *J. Clin. Microbiol.* **49:**111–117.

Hagens, S., and Loessner, M. J. (2010). Bacteriophage for biocontrol of foodborne pathogens: Calculations and considerations. *Curr. Pharm. Biotechnol.* **11:**58–68.

Hauben, K. J. L., Wuytack, E. Y., Soontjens, C. C. F., and Michiels, C. W. (1996). High-pressure transient sensitization of *Escherichia coli* to lysozyme and nisin by disruption of outer-membrane permeability. *J. Food Protection* **59:**350–355.

Hermoso, J. A., Garcia, J. L., and Garcia, P. (2007). Taking aim on bacterial pathogens: From phage therapy to enzybiotics. *Curr. Opin. Microbiol.* **10:**461–472.

Hermoso, J. A., Monterroso, B., Albert, A., Galan, B., Ahrazem, O., Garcia, P., Martinez-Ripoll, M., Garcia, J. L., and Menendez, M. (2003). Structural basis for selective recognition of pneumococcal cell wall by modular endolysin from phage Cp-1. *Structure* **11:**1239–1249.

Høiby, N., Bjarnsholt, T., Givskov, M., Molin, S., and Ciofu, O. (2010). Antibiotic resistance of bacterial biofilms. *Int. J. Antimicrob. Agents* **35**(4)**:**322–332.

Holtje, J. V., and Tomasz, A. (1975). Lipoteichoic acid: A specific inhibitor of autolysin activity in *Pneumococcus*. *Proc. Natl. Acad. Sci. USA* **72:**1690–1694.

Hoopes, J. T., Stark, C. J., Kim, H. A., Sussman, D. J., Donovan, D. M., and Nelson, D. C. (2009). Use of a bacteriophage lysin, PlyC, as an enzyme disinfectant against *Streptococcus equi*. *Appl. Environ. Microbiol.* **75:**1388–1394.

Horgan, M., O'Flynn, G., Garry, J., Cooney, J., Coffey, A., Fitzgerald, J. F., Ross, R. P., and McAuliffe, O. (2009). Phage lysin LysK can be truncated to its CHAP domain and retain lytic activity against live antibiotic-resistant staphylococci. *Appl. Environ. Microbiol.* **75:**872–874.

Huard, C., Miranda, G., Wessner, F., Bolotin, A., Hansen, J., Foster, S. J., and Chapot-Chartier, M. P. (2003). Characterization of AcmB, an N-acetylglucosaminidase autolysin from *Lactococcus lactis*. *Microbiology* **149:**695–705.

Imbuluzqueta, E., Gamazo, C., Ariza, J., and Blanco-Prieto, M. J. (2010). Drug delivery systems for potential treatment of intracellular bacterial infections. *Front. Biosci.* **15:**397–417.

Inouye, M., Arnheim, N., and Sternglanz, R. (1973). Bacteriophage T7 lysozyme is an N-acetylmuramyl-L-alanine amidase. *J Biol. Chem.* **248:**7247–7252.

Ito, Y., Kwon, O. H., Ueda, M., Tanaka, A., and Imanishi, Y. (1997). Bactericidal activity of human lysozymes carrying various lengths of polyproline chain at the C-terminus. *FEBS Lett.* **415:**285–288.

Jado, I., Lopez, R., Garcia, E., Fenoll, A., Casal, J., and Garcia, P. (2003). Phage lytic enzymes as therapy for antibiotic-resistant *Streptococcus pneumoniae* infection in a murine sepsis model. *J. Antimicrob. Chemother.* **52:**967–973.

Johnsborg, O., and Håvarstein, L. S. (2009). Regulation of natural genetic transformation and acquisition of transforming DNA in *Streptococcus pneumoniae*. *FEMS Microbiol. Rev.* **33:**627–642.

Joliot, A., and Prochiantz, A. (2004). Transduction peptides: From technology to physiology. *Nat. Cell Biol.* **6:**189–196.

Jones, R. N., Barry, A. L., Gavan, T. L., and Washington, J. A., II (1985). Susceptibility tests: Microdilution and macrodilution broth procedures. *In* ''Manual of Clinical Microbiology'' (A. Balows, J. W. J. Hausler, and H. J. Shadomy, eds.), pp. 972–977. American Society for Microbiology, Washington, DC.

Joris, B., Englebert, S., Chu, C. P., Kariyama, R., Neo-Moore, L., Shockman, G. D., and Ghuysen, J. M. (1992). Modular design of the *Enterococcus hirae* muramidase-2 and *Streptococcus faecalis* autolysin. *FEMS Microbiol. Lett.* **70:**257–264.

Kasparek, P., Pantucek, R., Kahankova, J., Ruzickova, V., and Doskar, J. (2007). Genome rearrangements in host-range mutants of the polyvalent staphylococcal bacteriophage 812. *Folia Microbiol. (Praha)* **52:**331–338.

Keren, I., Kaldalu, N., Spoering, A., Wang, Y., and Lewis, K. (2004). Persister cells and tolerance to antimicrobials. *FEMS Microbiol. Lett.* **230:**13–18.

Kerr, D. E., Plaut, K., Bramley, A. J., Williamson, C. M., Lax, A. J., Moore, K., Wells, K. D., and Wall, R. J. (2001). Lysostaphin expression in mammary glands confers protection against staphylococcal infection in transgenic mice. *Nat. Biotechnol.* **19:**66–70.

Kikkawa, H., Fujinami, Y., Suzuki, S., and Yasuda, J. (2007). Identification of the amino acid residues critical for specific binding of the bacteriolytic enzyme of gamma-phage, PlyG, to *Bacillus anthracis*. *Biochem. Biophys. Res. Commun.* **363:**531–535.

Kikkawa, H. S., Ueda, T., Suzuki, S., and Yasuda, J. (2008). Characterization of the catalytic activity of the gamma-phage lysin, PlyG, specific for *Bacillus anthracis*. *FEMS Microbiol. Lett.* **286:**236–240.

Kim, W. S., Salm, H., and Geider, K. (2004). Expression of bacteriophage phiEa1h lysozyme in *Escherichia coli* and its activity in growth inhibition of *Erwinia amylovora*. *Microbiology* **150:**2707–2714.

Kiri, N., Archer, G., and Climo, M. W. (2002). Combinations of lysostaphin with beta-lactams are synergistic against oxacillin-resistant *Staphylococcus epidermidis*. *Antimicrob. Agents Chemother.* **46:**2017–2020.

Kokai-Kun, J. F., Chanturiya, T., and Mond, J. J. (2007). Lysostaphin as a treatment for systemic *Staphylococcus aureus* infection in a mouse model. *J. Antimicrob. Chemother.* **60:**1051–1059.

Kokai-Kun, J. F., Chanturiya, T., and Mond, J. J. (2009). Lysostaphin eradicates established *Staphylococcus aureus* biofilms in jugular vein catheterized mice. *J. Antimicrob. Chemother.* **64:**94–100.

Kokai-Kun, J. F., Walsh, S. M., Chanturiya, T., and Mond, J. J. (2003). Lysostaphin cream eradicates *Staphylococcus aureus* nasal colonization in a cotton rat model. *Antimicrob. Agents Chemother.* **47:**1589–1597.

Korndorfer, I. P., Danzer, J., Schmelcher, M., Zimmer, M., Skerra, A., and Loessner, M. J. (2006). The crystal structure of the bacteriophage PSA endolysin reveals a unique fold responsible for specific recognition of *Listeria* cell walls. *J. Mol. Biol.* **364:**678–689.

Korndorfer, I. P., Kanitz, A., Danzer, J., Zimmer, M., Loessner, M. J., and Skerra, A. (2008). Structural analysis of the L-alanoyl-D-glutamate endopeptidase domain of *Listeria* bacteriophage endolysin Ply500 reveals a new member of the LAS peptidase family. *Acta Crystallogr. D Biol. Crystallogr.* **64:**644–650.

Krause, R. M. (1957). Studies on bacteriophages of hemolytic streptococci. *I. Factors influencing the interaction of phage and susceptible host cell. J Exp. Med.* **106:**365–384.

Kretzer, J. W., Lehmann, R., Schmelcher, M., Banz, M., Kim, K. P., Korn, C., and Loessner, M. J. (2007). Use of high-affinity cell wall-binding domains of bacteriophage endolysins for immobilization and separation of bacterial cells. *Appl. Environ. Microbiol.* **73:**1992–2000.

Kusuma, C., Jadanova, A., Chanturiya, T., and Kokai-Kun, J. F. (2007). Lysostaphin-resistant variants of *Staphylococcus aureus* demonstrate reduced fitness *in vitro* and *in vivo*. *Antimicrob. Agents Chemother.* **51:**475–482.

Kusuma, C., and Kokai-Kun, J. (2005). Comparison of four methods for determining lysostaphin susceptibility of various strains of *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **49:**3256–3263.

Lai, M. J., Lin, N. T., Hu, A., Soo, P. C., Chen, L. K., Chen, L. H., and Chang, K. C. (2011). Antibacterial activity of *Acinetobacter baumannii* phage ΦAB2 endolysin (LysAB2) against both Gram-positive and Gram-negative bacteria. *Appl. Microbiol. Biotechnol.* **90** (2)**:**529–539.

Lewis, K. (2001). Riddle of biofilm resistance. *Antimicrob Agents Chemother.* **45:**999–1007.
Lindsay, J. A. (2008). *Staphylococcus* Molecular Genetics. Caister Academic Press, Norfolk, UK.
Loeffler, J. M., Djurkovic, S., and Fischetti, V. A. (2003). Phage lytic enzyme Cpl-1 as a novel antimicrobial for pneumococcal bacteremia. *Infect. Immun.* **71:**6199–6204.
Loeffler, J. M., and Fischetti, V. A. (2003). Synergistic lethal effect of a combination of phage lytic enzymes with different activities on penicillin-sensitive and -resistant *Streptococcus pneumoniae* strains. *Antimicrob. Agents Chemother.* **47:**375–377.
Loeffler, J. M., Nelson, D., and Fischetti, V. A. (2001). Rapid killing of *Streptococcus pneumoniae* with a bacteriophage cell wall hydrolase. *Science* **294:**2170–2172.
Loessner, M. J. (2005). Bacteriophage endolysins: Current state of research and applications. *Curr. Opin. Microbiol.* **8:**480–487.
Loessner, M. J., Gaeng, S., Wendlinger, G., Maier, S. K., and Scherer, S. (1998). The two-component lysis system of *Staphylococcus aureus* bacteriophage Twort: A large TTG-start holin and an associated amidase endolysin. *FEMS Microbiol. Lett.* **162:**265–274.
Loessner, M. J., Kramer, K., Ebel, F., and Scherer, S. (2002). C-terminal domains of *Listeria monocytogenes* bacteriophage murein hydrolases determine specific recognition and high-affinity binding to bacterial cell wall carbohydrates. *Mol. Microbiol.* **44:**335–349.
Loessner, M. J., Schneider, A., and Scherer, S. (1995a). A new procedure for efficient recovery of DNA, RNA, and proteins from *Listeria* cells by rapid lysis with a recombinant bacteriophage endolysin. *Appl. Environ. Microbiol.* **61:**1150–1152.
Loessner, M. J., Wendlinger, G., and Scherer, S. (1995b). Heterogeneous endolysins in *Listeria monocytogenes* bacteriophages: A new class of enzymes and evidence for conserved holin genes within the siphoviral lysis cassettes. *Mol. Microbiol* **16:**1231–1241.
Lopez, R., Garcia, E., Garcia, P., and Garcia, J. L. (1997). The pneumococcal cell wall degrading enzymes: a modular design to create new lysins? *Microb. Drug. Resist.* **3:**199–211.
Lopez, R., Garcia, E., Garcia, P., Ronda, C., and Tomasz, A. (1982). Choline-containing bacteriophage receptors in *Streptococcus pneumoniae*. *J. Bacteriol.* **151:**1581–1590.
Low, L. Y., Yang, C., Perego, M., Osterman, A., and Liddington, R. C. (2005). Structure and lytic activity of a *Bacillus anthracis* prophage endolysin. *J. Biol. Chem.* **280:**35433–35439.
Lu, J. Z., Fujiwara, T., Komatsuzawa, H., Sugai, M., and Sakon, J. (2006). Cell wall-targeting domain of glycyl-glycine endopeptidase distinguishes among peptidoglycan cross-bridges. *J. Biol. Chem.* **281:**549–558.
Maga, E. A., Cullor, J. S., Smith, W., Anderson, G. B., and Murray, J. D. (2006a). Human lysozyme expressed in the mammary gland of transgenic dairy goats can inhibit the growth of bacteria that cause mastitis and the cold-spoilage of milk. *Foodborne. Pathog. Dis.* **3:**384–392.
Maga, E. A., Walker, R. K., Anderson, G. B., and Murray, J. D. (2006b). Consumption of milk from transgenic goats expressing human lysozyme in the mammary gland results in the modulation of intestinal microflora. *Transgenic Res.* **15:**515–519.
Manoharadas, S., Witte, A., and Blasi, U. (2009). Antimicrobial activity of a chimeric enzybiotic towards *Staphylococcus aureus*. *J. Biotechnol.* **139:**118–123.
Masschalck, B., Garcia-Graells, C., Van Haver, E., and Michiels, C. W. (2000). Inactivation of high pressure resistant *Escherichia coli* by nisin and lysozyme under high pressure. *Innov. Food Sci. Emerg. Technol.* **1:**39.
Masschalck, B., and Michiels, C. W. (2003). Antimicrobial properties of lysozyme in relation to foodborne vegetative bacteria. *Crit. Rev. Microbiol.* **29:**191–214.
Masschalck, B., Van, H. R., and Michiels, C. W. (2001). High pressure increases bactericidal activity and spectrum of lactoferrin, lactoferricin and nisin. *Int. J. Food Microbiol.* **64:**325–332.
Mayer, M. J., Narbad, A., and Gasson, M. J. (2008). Molecular characterization of a *Clostridium difficile* bacteriophage and its cloned biologically active endolysin. *J. Bacteriol.* **190:**6734–6740.

Mayer, M. J., Payne, J., Gasson, M. J., and Narbad, A. (2010). Genomic sequence and characterization of the virulent bacteriophage phiCTP1 from *Clostridium tyrobutyricum* and heterologous expression of its endolysin. *Appl. Environ. Microbiol.* **76:**5415–5422.

McCullers, J. A., Karlstrom, A., Iverson, A. R., Loeffler, J. M., and Fischetti, V. A. (2007). Novel strategy to prevent otitis media caused by colonizing *Streptococcus pneumoniae*. *PLoS Pathog.* **3:**e28.

Mesnage, S., Chau, F., Dubost, L., and Arthur, M. (2008). Role of N-acetylglucosaminidase and N-acetylmuramidase activities in *Enterococcus faecalis* peptidoglycan metabolism. *J. Biol. Chem.* **283:**19845–19853.

Mitchell, G. J., Nelson, D. C., and Weitz, J. S. (2010). Quantifying enzymatic lysis: Estimating the combined effects of chemistry, physiology and physics. *Phys. Biol.* **7:**046002.

Moak, M., and Molineux, I. J. (2004). Peptidoglycan hydrolytic activities associated with bacteriophage virions. *Mol. Microbiol.* **51:**1169–1183.

Mokrasch, L. C. (1967). Use of 2,4,6-trinitrobenzenesulfonic acid for the coestimation of amines, amino acids and proteins in mixtures. *Anal. Biochem* **18:**64–71.

Monterroso, B., Saiz, J. L., Garcia, P., Garcia, J. L., and Menendez, M. (2008). Insights into the structure-function relationships of pneumococcal cell wall lysozymes, LytC and Cpl-1. *J. Biol. Chem.* **283:**28618–28628.

Morita, M., Tanji, Y., Orito, Y., Mizoguchi, K., Soejima, A., and Unno, H. (2001). Functional analysis of antibacterial activity of *Bacillus amyloliquefaciens* phage endolysin against Gram-negative bacteria. *FEBS Lett.* **500:**56–59.

Muyombwe, A., Tanji, Y., and Unno, H. (1999). Cloning and expression of a gene encoding the lytic functions of *Bacillus amyloliquefaciens* phage: Evidence of an auxiliary lysis system. *J. Biosci. Bioeng.* **88:**221–225.

Nakimbugwe, D., Masschalck, B., Deckers, D., Callewaert, L., Aertsen, A., and Michiels, C. W. (2006). Cell wall substrate specificity of six different lysozymes and lysozyme inhibitory activity of bacterial extracts. *FEMS Microbiol. Lett.* **259:**41–46.

Navarre, W. W., Ton-That, H., Faull, K. F., and Schneewind, O. (1999). Multiple enzymatic activities of the murein hydrolase from staphylococcal phage phi11: Identification of a D-alanyl-glycine endopeptidase activity. *J. Biol. Chem.* **274:**15847–15856.

Nelson, D., Loomis, L., and Fischetti, V. A. (2001). Prevention and elimination of upper respiratory colonization of mice by group A streptococci by using a bacteriophage lytic enzyme. *Proc. Natl. Acad. Sci. USA* **98:**4107–4112.

Nelson, D., Schuch, R., Chahales, P., Zhu, S., and Fischetti, V. A. (2006). PlyC: A multimeric bacteriophage lysin. *Proc. Natl. Acad. Sci. USA* **103:**10765–10770.

Nelson, D., Schuch, R., Zhu, S., Tscherne, D. M., and Fischetti, V. A. (2003). Genomic sequence of C1, the first streptococcal phage. *J Bacteriol.* **185:**3325–3332.

Obeso, J. M., Martinez, B., Rodriguez, A., and Garcia, P. (2008). Lytic activity of the recombinant staphylococcal bacteriophage PhiH5 endolysin active against *Staphylococcus aureus* in milk. *Int. J. Food Microbiol.* **128:**212–218.

Oey, M., Lohse, M., Kreikemeyer, B., and Bock, R. (2009). Exhaustion of the chloroplast protein synthesis capacity by massive expression of a highly stable protein antibiotic. *Plant J.* **57:**436–445.

O'Flaherty, S., Coffey, A., Edwards, R., Meaney, W., Fitzgerald, G. F., and Ross, R. P. (2004). Genome of staphylococcal phage K: A new lineage of *Myoviridae* infecting gram-positive bacteria with a low G+C content. *J. Bacteriol.* **186:**2862–2871.

O'Flaherty, S., Coffey, A., Meaney, W., Fitzgerald, G. F., and Ross, R. P. (2005). The recombinant phage lysin LysK has a broad spectrum of lytic activity against clinically relevant staphylococci, including methicillin-resistant *Staphylococcus aureus*. *J. Bacteriol.* **187:**7161–7164.

O'Flaherty, S., Ross, R. P., and Coffey, A. (2009). Bacteriophage and their lysins for elimination of infectious bacteria. *FEMS Microbiol. Rev.* **33:**801–819.

Oldham, E. R., and Daley, M. J. (1991). Lysostaphin: Use of a recombinant bactericidal enzyme as a mastitis therapeutic. *J. Dairy Sci.* **74:**4175–4182.

Orito, Y., Morita, M., Hori, K., Unno, H., and Tanji, Y. (2004). *Bacillus amyloliquefaciens* phage endolysin can enhance permeability of *Pseudomonas aeruginosa* outer membrane and induce cell lysis. *Appl. Microbiol. Biotechnol.* **65:**105–109.

Palmer, K. L., Kos, V. N., and Gilmore, M. S. (2010). Horizontal gene transfer and the genomics of enterococcal antibiotic resistance. *Curr. Opin. Microbiol.* **13:**632–639.

Pangule, R. C., Brooks, S. J., Dinu, C. Z., Bale, S. S., Salmon, S. L., Zhu, G., Metzger, D. W., Kane, R. S., and Dordick, J. S. (2010). Antistaphylococcal nanocomposite films based on enzyme-nanotube conjugates. *ACS Nano.* **4:**3993–4000.

Paradis-Bleau, C., Cloutier, I., Lemieux, L., Sanschagrin, F., Laroche, J., Auger, M., Garnier, A., and Levesque, R. C. (2007). Peptidoglycan lytic activity of the *Pseudomonas aeruginosa* phage phiKZ gp144 lytic transglycosylase. *FEMS Microbiol. Lett.* **266:**201–209.

Park, J. T., and Johnson, M. J. (1949). A submicrodetermination of glucose. *J. Biol. Chem.* **181:**149–151.

Pastagia, M., Euler, C., Chahales, P., Fuentes-Duculan, J., Krueger, J. G., and Fischetti, V. A. (2011). A novel chimeric lysin shows superiority to mupirocin for skin decolonization of methicillin-resistant and -sensitive *Staphylococcus aureus* strains. *Antimicrob. Agents Chemother.* **55:**738–744.

Patron, R. L., Climo, M. W., Goldstein, B. P., and Archer, G. L. (1999). Lysostaphin treatment of experimental aortic valve endocarditis caused by a *Staphylococcus aureus* isolate with reduced susceptibility to vancomycin. *Antimicrob. Agents Chemother.* **43:**1754–1755.

Ponting, C. P., Aravind, L., Schultz, J., Bork, P., and Koonin, E. V. (1999). Eukaryotic signalling domain homologues in archaea and bacteria: Ancient ancestry and horizontal gene transfer. *J. Mol. Biol.* **289:**729–745.

Porter, C. J., Schuch, R., Pelzek, A. J., Buckle, A. M., McGowan, S., Wilce, M. C., Rossjohn, J., Russell, R., Nelson, D., Fischetti, V. A., and Whisstock, J. C. (2007). The 1.6 A crystal structure of the catalytic domain of PlyB, a bacteriophage lysin active against *Bacillus anthracis*. *J. Mol. Biol.* **366:**540–550.

Pritchard, D. G., Dong, S., Baker, J. R., and Engler, J. A. (2004). The bifunctional peptidoglycan lysin of *Streptococcus agalactieae* bacteriophage B30. *Microbiology* **150:**2079–2087.

Pritchard, D. G., Dong, S., Kirk, M. C., Cartee, R. T., and Baker, J. R. (2007). LambdaSa1 and LambdaSa2 prophage lysins of *Streptococcus agalactiae*. *Appl. Environ. Microbiol.* **73:**7150–7154.

Rashel, M., Uchiyama, J., Ujihara, T., Uehara, Y., Kuramoto, S., Sugihara, S., Yagyu, K., Muraoka, A., Sugai, M., Hiramatsu, K., Honke, K., and Matsuzaki, S. (2007). Efficient elimination of multidrug-resistant *Staphylococcus aureus* by cloned lysin derived from bacteriophage phi MR11. *J. Infect. Dis.* **196:**1237–1247.

Rigden, D. J., Jedrzejas, M. J., and Galperin, M. Y. (2003). Amidase domains from bacterial and phage autolysins define a family of gamma-D,L-glutamate-specific amidohydrolases. *Trends Biochem. Sci.* **28:**230–234.

Rossolini, G. M., Mantengoli, E., Montagnani, F., and Pollini, S. (2010). Epidemiology and clinical relevance of microbial resistance determinants versus anti-Gram-positive agents. *Curr. Opin. Microbiol.* **13:**582–588.

Sahl, H. G., and Bierbaum, G. (2008). Multiple activities in natural antimicrobials. *Microbe* **3:**467–473.

Sanz, J. M., Garcia, P., and Garcia, J. L. (1996). Construction of a multifunctional pneumococcal murein hydrolase by module assembly. *Eur. J. Biochem.* **235:**601–605.

Sass, P., and Bierbaum, G. (2007). Lytic activity of recombinant bacteriophage phi11 and phi12 endolysins on whole cells and biofilms of *Staphylococcus aureus*. *Appl. Environ. Microbiol.* **73:**347–352.

Satake, K., Okuyama, T., Ohashi, M., and Shinoda, T. (1960). The spectrophotometric determination of amine, amino acid and peptide with 2,4,6,-trinitrobenzene 1-sulfonic acid. *J. Biochem.* **47:**654–660.

Sawhney, R., and Berry, V. (2009). Bacterial biofilm formation, pathogenicity, diagnostics and control: An overview. *Indian J. Med. Sci.* **63:**313–321.

Schaffner, W., Melly, M. A., and Koenig, M. G. (1967). Lysostaphin: An enzymatic approach to staphylococcal disease. *II. In vivo studies. Yale J. Biol. Med.* **39:**230–244.

Scheurwater, E. M., and Clarke, A. J. (2008). The C-terminal domain of *Escherichia coli* YfhD functions as a lytic transglycosylase. *J. Biol. Chem.* **283:**8363–8373.

Schindler, C. A., and Schuhardt, V. T. (1964). Lysostaphin: A new bacteriolytic agent for the *Staphylococcus*. *Proc. Natl. Acad. Sci. USA* **51:**414–421.

Schleifer, K. H., and Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol. Rev.* **36:**407–477.

Schmelcher, M., Shabarova, T., Eugster, M. R., Eichenseher, F., Tchang, V. S., Banz, M., and Loessner, M. J. (2010). Rapid multiplex detection and differentiation of *Listeria* cells by use of fluorescent phage endolysin cell wall binding domains. *Appl. Environ. Microbiol.* **76:**5745–5756.

Schmelcher, M., Tchang, V. S., and Loessner, M. J. (2011). Domain shuffling and module engineering of *Listeria* phage endolysins for enhanced lytic activity and binding affinity. *Microb. Biotechnol.* **4:**651–652.

Schuch, R., Fischetti, V. A., and Nelson, D. C. (2009). A genetic screen to identify bacteriophage lysins. *Methods Mol. Biol.* **502:**307–319.

Schuch, R., Nelson, D., and Fischetti, V. A. (2002). A bacteriolytic agent that detects and kills *Bacillus anthracis*. *Nature* **418:**884–889.

Seltman, G., and Holst, O. (2001). ''The Bacterial Cell Wall''. Springer Verlag, Berlin.

Sheehan, M. M., Garcia, J. L., Lopez, R., and Garcia, P. (1996). Analysis of the catalytic domain of the lysin of the lactococcal bacteriophage Tuc 2009 by chimeric gene assembling. *FEMS Microbiol. Lett.* **140:**23–28.

Silva-Martin, N., Molina, R., Angulo, I., Mancheno, J. M., Garcia, P., and Hermoso, J. A. (2010). Crystallization and preliminary crystallographic analysis of the catalytic module of endolysin from Cp-7, a phage infecting *Streptococcus pneumoniae*. *Acta Crystallogr. F Struct. Biol. Cryst. Commun.* **66:**670–673.

Simmons, M., Donovan, D. M., Siragusa, G. R., and Seal, B. S. (2010). Recombinant expression of two bacteriophage proteins that lyse *Clostridium perfringens* and share identical sequences in the C-terminal cell wall binding domain of the molecules but are dissimilar in their N-terminal active domains. *J. Agric. Food Chem.* **58:**10330–10337.

Simões, M. (2011). Antimicrobial strategies effective against infectious bacterial biofilms. *Curr. Med. Chem.* **18:**2129–2145.

Son, J. S., Lee, S. J., Jun, S. Y., Yoon, S. J., Kang, S. H., Paik, H. R., Kang, J. O., and Choi, Y. J. (2010). Antibacterial and biofilm removal activity of a podoviridae *Staphylococcus aureus* bacteriophage SAP-2 and a derived recombinant cell-wall-degrading enzyme. *Appl. Microbiol. Biotechnol.* **86:**1439–1449.

Spiro, R. G. (1966). Analysis of sugars found in glycoproteins. *Methods Enzymol.* **8:**3–26.

Splith, K., and Neundorf, I. (2011). Antimicrobial peptides with cell-penetrating peptide properties and vice versa. *Eur. Biophys. J.* **40**(4)**:**387–397.

Stark, C. J., Hoopes, J. T., Bonocoroa, R. P., and Nelson, D. C. (2010). Bacteriophage lytic enzymes as antimicrobials. *In* ''Bacteriophage in the Detection and Control of Foodborne Pathogens'' (P. V. Sabour and M. W. Griffith, eds.), pp. 137–156. ASM Press, Washington, DC.

Steen, A., van Schalkwijk, S., Buist, G., Twigt, M., Szeliga, W., Meijer, W., Kuipers, O. P., Kok, J., and Hugenholz, J. (2007). Lytr, a phage-derived amidase is most effective in

induced lysis of *Lactococcus lactis* compared with other lactococcal amidases and glucosaminidases. *Int. Dairy J.* **17:**926–936.
Stentz, C. R., Bongaerts, R. J., Gunning, A. P., Gasson, M., and Shearman, C. (2010). Controlled release of protein from viable *Lactococcus lactis* cells. *Appl. Environ. Microbiol.* **76:**3026–3031.
Sugai, M., Fujiwara, T., Ohta, K., Komatsuzawa, H., Ohara, M., and Suginaka, H. (1997). epr, which encodes glycylglycine endopeptidase resistance, is homologous to *femAB* and affects serine content of peptidoglycan cross bridges in *Staphylococcus capitis* and *Staphylococcus aureus*. *J. Bacteriol.* **179:**4311–4318.
Sun, H. C., Xue, F. M., Qian, K., Fang, X. H., Qiu, H. L., Zhang, X. Y., and Yin, Z. H. (2006). Intramammary expression and therapeutic effect of a human lysozyme-expressing vector for treating bovine mastitis. *J. Zhejiang. Univ. Sci. B* **7:**324–330.
Tan, Y. P., Giffard, P. M., Barry, D. G., Huston, W. M., and Turner, M. S. (2008). Random mutagenesis identifies novel genes involved in the secretion of antimicrobial, cell wall-lytic enzymes by *Lactococcus lactis*. *Appl. Environ. Microbiol.* **74:**7490–7496.
Taylor, A., and Gorazdowska, M. (1974). Conversion of murein to non-reducing fragments by enzymes from phage lambda and Vi II lysates. *Biochim. Biophys. Acta* **342:**133–136.
Thumm, G., and Gotz, F. (1997). Studies on prolysostaphin processing and characterization of the lysostaphin immunity factor (Lif) of *Staphylococcus simulans* biovar *staphylolyticus*. *Mol. Microbiol.* **23:**1251–1265.
Tong, J., Wei, H., Liu, X., Hu, W., Bi, M., Wang, Y., Li, Q., and Li, N. (2010). Production of recombinant human lysozyme in the milk of transgenic pigs. *Transgenic Res.* **20** (2)**:**417–419.
Turner, M. S., Waldherr, F., Loessner, M. J., and Giffard, P. M. (2007). Antimicrobial activity of lysostaphin and a *Listeria monocytogenes* bacteriophage endolysin produced and secreted by lactic acid bacteria. *Syst. Appl. Microbiol.* **30:**58–67.
Vaara, M. (1992). Agents that increase the permeability of the outer membrane. *Microbiol. Rev.* **56:**395–411.
Vasala, A., Valkkila, M., Caldentey, J., and Alatossava, T. (1995). Genetic and biochemical characterization of the *Lactobacillus delbrueckii* subsp. *lactis* bacteriophage LL-H lysin. *Appl. Environ. Microbiol.* **61:**4004–4011.
Vazquez-Boland, J. A., Kuhn, M., Berche, P., Chakraborty, T., Dominguez-Bernal, G., Goebel, W., Gonzalez-Zorn, B., Wehland, J., and Kreft, J. (2001). *Listeria* pathogenesis and molecular virulence determinants. *Clin. Microbiol. Rev.* **14:**584–640.
Vollmer, W. (2008). Structural variation in the glycan strands of bacterial peptidoglycan. *FEMS Microbiol. Rev.* **32:**287–306.
Walcher, G., Stessl, B., Wagner, M., Eichenseher, F., Loessner, M. J., and Hein, I. (2010). Evaluation of paramagnetic beads coated with recombinant Listeria phage endolysin-derived cell-wall-binding domain proteins for separation of *Listeria monocytogenes* from raw milk in combination with culture-based and real-time polymerase chain reaction-based quantification. *Foodborne Pathog. Dis.* **7:**1019–1024.
Walencka, E., Sadowska, B., Rozalska, S., Hryniewicz, W., and Rozalska, B. (2005). Lysostaphin as a potential therapeutic agent for staphylococcal biofilm eradication. *Pol. J. Microbiol.* **54:**191–200.
Wall, R. J., Powell, A., Paape, M. J., Kerr, D. E., Bannerman, D. D., Pursel, V. G., Wells, K. D., Talbot, N., and Hawk, H. W. (2005). Genetically enhanced cows resist intramammary *Staphylococcus aureus* infection. *Nat. Biotechnol.* **23:**445–451.
Ward, J. B. (1973). The chain length of the glycans in bacterial cell walls. *Biochem. J.* **133:**395–398.
Weidel, W., and Pelzer, H. (1964). Bagshaped macromolecules: A new outlook on bacterial cell walls. *Adv. Enzymol. Relat. Areas Mol. Biol.* **26:**193–232.

Whisstock, J. C., and Lesk, A. M. (1999). SH3 domains in prokaryotes. *Trends Biochem. Sci.* **24:**132–133.

Wu, J. A., Kusuma, C., Mond, J. J., and Kokai-Kun, J. F. (2003). Lysostaphin disrupts *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms on artificial surfaces. *Antimicrob. Agents Chemother.* **47:**3407–3414.

Yang, B., Wang, J., Tang, B., Liu, Y., Guo, C., Yang, P., Yu, T., Li, R., Zhao, J., Zhang, L., Dai, Y., and Li, N. (2011). Characterization of bioactive recombinant human lysozyme expressed in milk of cloned transgenic cattle. *PLoS One* **6:**e17593.

Yang, G., Gao, Y., Feng, J., Huang, Y., Li, S., Liu, Y., Liu, C., Fan, M., Shen, B., and Shao, N. (2008). C-terminus of TRAP in *Staphylococcus* can enhance the activity of lysozyme and lysostaphin. *Acta Biochim. Biophys. Sin. (Shanghai)* **40:**452–458.

Yoong, P., Schuch, R., Nelson, D., and Fischetti, V. A. (2004). Identification of a broadly active phage lytic enzyme with lethal activity against antibiotic-resistant *Enterococcus faecalis* and *Enterococcus faecium*. *J. Bacteriol.* **186:**4808–4812.

Yoong, P., Schuch, R., Nelson, D., and Fischetti, V. A. (2006). PlyPH, a bacteriolytic enzyme with a broad pH range of activity and lytic action against *Bacillus anthracis*. *J. Bacteriol.* **188:**2711–2714.

Young, R. (1992). Bacteriophage lysis: Mechanism and regulation. *Microbiol Rev.* **56:**430–481.

Zimmer, M., Vukov, N., Scherer, S., and Loessner, M. J. (2002). The murein hydrolase of the bacteriophage phi3626 dual lysis system is active against all tested *Clostridium perfringens* strains. *Appl. Environ. Microbiol.* **68:**5311–5317.