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Bacteriophage endolysins as novel antimicrobials

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Abstract

Endolysins are enzymes used by bacteriophages at the end of their replication cycle to degrade the peptidoglycan of the bacterial host from within, resulting in cell lysis and release of progeny virions. Due to the absence of an outer membrane in the Gram-positive bacterial cell wall, endolysins can access the peptidoglycan and destroy these organisms when applied externally, making them interesting antimicrobial candidates, particularly in light of increasing bacterial drug resistance. This article reviews the modular structure of these enzymes, in which cell wall binding and catalytic functions are separated, as well as their mechanism of action, lytic activity and potential as antimicrobials. It particularly focuses on molecular engineering as a means of optimizing endolysins for specific applications, highlights new developments that may render these proteins active against Gram-negative and intracellular pathogens and summarizes the most recent applications of endolysins in the fields of medicine, food safety, agriculture and biotechnology.

Keywords

antimicrobial; bacteriophage; detection; endolysin; lysis; peptidoglycan hydrolase; protein engineering

Endolysins, also termed phage lysins, are phage-encoded peptidoglycan hydrolases (PGHs) employed by the majority of bacteriophages to enzymatically degrade the peptidoglycan (PG) layer of the host bacterium `from within' at the end of their lytic multiplication cycle. PG functions as the major structural component of the bacterial cell, supporting an internal turgor pressure of 20–50 atmospheres in the case of Gram-positive organisms [1–3]. Breach of the PG layer results in osmotic lysis and cell death of the bacterium, enabling liberation of progeny virions. Host cell lysis is mostly strictly regulated and exactly timed with the help of a holin, which constitutes the second component of the lysis cassette of tailed phages. Holins are produced during the late stages of infection and, once a critical concentration is

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reached, create holes in the cytoplasmic membrane by oligomerization, allowing the endolysins, which have accumulated in the cytoplasm, to access their PG substrate [4,5]. Alternative ways of regulating the lysis event (not within the scope of this article) have been described, such as those involving endolysins with signal peptides, N-terminal signal arrest release sequences or proteins inhibiting PG synthesis ([6–8], reviewed in [9]), and filamentous phages achieve liberation of progeny by an entirely different mechanism without killing their hosts [10]. For the purpose of this review, it is important that endolysins are also capable of killing susceptible organisms when applied exogenously as recombinant proteins. For this reason, they have attracted increasing interest as potential antimicrobial agents, particularly in light of emerging and spreading resistance of bacteria against classical antibiotics. Fischetti and coworkers coined the term `enzybiotics' for these proteins, describing both their enzymatic and antibacterial properties [11]. `Lysis from without' caused by purified endolysins should not be confused with the lytic action of phage structural proteins, which are usually tail-associated components of the virion that locally degrade the PG from without after attachment of the phage particle to the cell in order to enable injection of bacteriophage DNA into the host [12–14]. This was reported in 1940 when a high multiplicity of infection (phage:host ratio) yielded a reduction in culture turbidity well below the time required for the phage lytic cycle [15]. Historically, application of endolysins as antimicrobials has been limited to Gram-positive pathogens, whose PG is not protected from the environment by an outer membrane (OM), as is the case with Gram-negative species. However, recent developments involving peptides with OM-disrupting properties fused to phage lysins have raised hopes of also tackling Gram-negative organisms with PGH enzymes. This will be discussed in this review. An important advantage of endolysins over classical antibiotics is their high specificity for certain PG types, which generally limits their antimicrobial action to members of a certain bacterial genus, species or even serotype [16,17]. This near-species specificity greatly reduces the risk of resistant (commensal) strain development that is often associated with the use of broad-range antibiotics, allowing for selective killing of given target pathogens, with commensal bacteria or desired organisms of the accompanying microflora being unaffected [18].

Phage endolysins are similar in structure and function to bacterial auto- and exo-lysins [19,20], and also closely related to the small family of mammalian PG recognition proteins [21]. While autolysins are mainly involved in PG remodeling during cell growth and division [22], bacterial exolysins are produced and secreted by a bacterium in order to kill cells of a different strain or species. A prominent example for the latter class is lysostaphin, a bacteriocin produced by *Staphylococcus simulans* that exhibits lytic activity against *Staphylococcus aureus* [23]. The antibacterial potential of lysostaphin, as well as bacterial autolysins, has been demonstrated in a multitude of studies, partly involving animal models (reviewed in [20]). There have been numerous reviews of PGHs as antimicrobials, often including exolysins, bacteriocins and phage endolysins [9,18,20,24–31]. However, this review will primarily focus on bacteriophage endolysins and their structure and function, as well as applications in different fields for the detection and control of bacterial pathogens. In particular, the high potential of molecular engineering as a means of tailoring endolysin properties for specific applications will be discussed.

Endolysin structure & mechanism of action

The structure of phage endolysins generally differs between those enzymes targeting Gram-positive and Gram-negative bacteria, reflecting the differences in cell wall architecture between these major bacterial groups. Endolysins from a Gram-positive background have evolved to utilize a modular design in which catalytic activity and substrate recognition are separated into two distinct types of functional domains termed cell wall binding domains (CBDs) and enzymatically active domains (EADs) [9,16,18,25,32]. While the EAD confers

the catalytic mechanism of the enzyme (i.e., cleaving specific bonds within the bacterial PG), the CBD has been shown, for some lysins, to target the protein to its substrate and to keep it tightly bound to cell wall debris after cell lysis, thereby likely preventing diffusion and subsequent destruction of surrounding intact cells that have not yet been infected by the phage [33]. By contrast, the OM of Gram-negative organisms prevents this sort of collateral damage by limiting access to the PG from the outside. This might explain why endolysins from phages infecting Gram-negative hosts are mostly small single-domain globular proteins (molecular mass between 15 and 20 kDa), usually without a specific CBD module [34,35]. These lysins likely better fulfill the catalytic role of classical enzymes (aiding multiple catalytic reactions during cell lysis), as opposed to their Gram-positive counterparts, which are proposed to bind to one site and have a very low off-rate [17,33] [Eichenseher F et al. Unpublished Data]. However, for every rule, there are always exceptions. Two Gram-negative lysins from *Pseudomonas* phages (KZ144 and EL188) have been reported to harbor a modular architecture with an N-terminal CBD and a C-terminal EAD. The N-terminal portion of KZ144 binds to *Pseudomonas aeruginosa* cell walls with high affinity and recognizes PG of other Gram-negative bacteria [35,36]. Gram-positive endolysins, on the other hand, mostly feature an opposite orientation of functional modules, with an EAD at the N-terminus and a CBD at the C-terminus in the most simple case (e.g., as observed in lysins from phages infecting *Listeria*, *Clostridium* and *Bacillus* [16,17,37–41]). However, endolysin structures are not necessarily limited to only two modules, and the different architectures and domain orientations found in public databases are manifold (Figure 1) (reviewed in [26]). Prominent examples include the combination of two N-terminal EADs and one C-terminal CBD, as frequently found in staphylococcal lysins [42–46], centrally located CBDs separating two terminal EADs, as in the streptococcal phage λSA2 endolysin [47], and CBDs consisting of up to 18 tandem repeats of an approximately 20-amino acid motif, characteristic of phage lysins that target the choline-containing teichoic acids of *Streptococcus pneumoniae* [31,32,48]. While almost all Gram-positive endolysins described to date are encoded by a single gene, the PlyC endolysin of the group A *Streptococcus* phage C1 is the only example of a multimeric phage lysin composed of two different gene products, PlyCA and PlyCB [49]. One PlyCA subunit harboring the enzymatic activity and eight PlyCB subunits constituting the CBD form the complete PlyC complex, which is the endolysin with the highest activity reported to date, with nanogram quantities able to clear a turbid culture within seconds [49,50].

Enzymatically active domains

The EAD embodies the lytic mechanism of an endolysin, catalyzing the breakdown of the PG. Endolysins can be classified into at least five different groups depending on the specific bond of the PG attacked by the EAD: *N*-acetyl-β-D-muramidases (generically also termed lysozymes) and lytic transglycosylases, both of which cleave the *N*-acetylmuramoyl-β-1,4-*N*-acetylglucosamine bond, which is one of the two alternating glycosidic bonds of the sugar strand; *N*-acetyl-β-D-glucosaminidases cut the other glycosidic bond (*N*-acetylglucosaminyl-β-1,4-*N*-acetylmuramine) in the sugar strand; *N*-acetylmuramoyl-L-alanine amidases hydrolyze the amide bond between the sugar and the peptide moieties; and endopeptidases cleave within the peptides making up the interconnecting stem portion of the PG units (Figure 2) (for extensive reviews on bacterial PG types, see [51,52]). According to the International Union of Biochemistry and Molecular Biology (IUBMB) enzyme nomenclature [301], both muramidases and glucosamininidases are glycosidases, and all endolysins except for the lytic transglycosylases are hydrolases (i.e., they require a water molecule for catalyzing the reaction). The transglycosylases cleave the β-1,4-glycosidic bond by an entirely different mechanism, involving an intramolecular reaction that results in the formation of a 1,6-anhydro ring at the MurNAc residue [53,54]. Endopeptidases can be further divided into enzymes that cut within the stem peptide of the PG and those that attack

bonds within the interpeptide bridge (if present), such as the pentaglycine bridge in *S. aureus* PG. *N*-acetylmuramoyl-L-alanine amidases are among the most frequently and earliest identified PGHs. The abbreviation of this term and its subsequent overuse to describe all PGHs (in the absence of biochemical evidence for the actual enzymatic activity conferred by the enzymatic domain) has led to quite some confusion over endolysin nomenclature, with endopeptidases that were eventually biochemically verified having been frequently referred to previously as `amidases' in public databases. For the sake of clarity, we use the term `amidase' exclusively for those amidases that hydrolyze nonpeptide bonds, that is *N*-acetylmuramoyl-L-alanine amidases. Furthermore, databases contain terms specifying families of conserved EADs, such as the CHAP domain [55,56], which refers to the molecular mechanism by which the cleavage occurs rather than the specific bond cleaved. Some CHAP domains have been reported to have amidase activity [49], while others are endopeptidases [57,58]. Similar to the term `amidase', ambiguous usage of the generic term `lysozyme' can also be found in the literature and public databases, with enzymes being designated lysozymes that have been shown experimentally to exhibit other sugar strand-cleaving or even amidase activity, such as for the λ and T7 phage endolysins, respectively [59,60]. While assignment of cleavage specificity to EADs of newly discovered endolysins through bioinformatic analysis (i.e., based on sequence homology with other domains) is the most commonly used method, it can yield erroneous results that can readily be propagated within the databases. By contrast, there are numerous experimental ways to unambiguously determine an enzyme's catalytic specificity, including various classical biochemical methods, N-terminal sequencing of digestion products and mass spectrometry (reviewed in [26]). It should be noted that EADs can also contribute to the specificity of an endolysin for certain genera or species of bacteria, based on the presence or absence of that EAD's specific target bond in the respective PG types. That being said, amidases and muramidases are not only the most commonly found enzymes [9], but are also among the most universal ones, targeting highly conserved bonds in the PG. This scenario is further complicated by the fact that the activity of some PGHs (e.g., glycosidases) can be further modulated by secondary modifications of the PG such as *O*-acetylation or *N*-deacetylation (reviewed in [61]). Moreover, as the cell walls of Gram-positive bacteria are generally negatively charged, the net charge of an EAD might also play a role in determining its lytic activity and host range [21].

Cell wall binding domains

CBDs confer specificity to an endolysin for certain cell wall types by recognizing and binding (noncovalently) to ligand molecules within the cell envelope (which may be parts of the PG itself or other cell wall-associated molecules), thereby significantly impacting the activity range of the enzyme. Various conserved binding modules have been described in the literature such as: the LysM domain (reviewed in [62]), which is reportedly the most common domain in PGHs and for at least some enzymes and has been shown to bind to GlcNAc residues in the sugar backbone of the PG [63]; the bacterial SH3b domain [64,65]; the choline binding modules of Cpl-1 and other pneumococcal lysins [31,32,66], which specifically recognize the choline-containing teichoic acids in the cell wall of *S. pneumoniae*; and the Cpl-7 binding domain, which binds to pneumococcal cell walls in a choline-independent fashion [67,68] and, interestingly, is also present in tandem in the streptococcal λSA2 lysin [47]. The binding spectrum of a CBD in many cases encompasses an entire bacterial genus, as observed in binding studies with various GFP-tagged staphylococcal SH3b binding domains [69] [Eichenseher F *et al.* Unpublished Data], and is therefore generally broader than the host range of the respective phage. This indicates recognition of a rather conserved binding ligand such as the glycine-rich interpeptide bridge common to most staphylococcal strains [52], as has been reported for the SH3b-like cell wall targeting domain of lysostaphin [70]. Examples of endolysins with even broader

activity ranges exist, such as the enterococcal PlyV12 endolysin, which kills not only enterococci but also numerous streptococcal and staphylococcal strains [71]. However, the study did not investigate whether the binding spectrum of the putative CBD of PlyV12 is consistent with this very broad range of activity. On the other hand, binding modules can exhibit specificity down to the serovar or even strain level, as reported for CBDs of *Listeria* phage endolysins, which are mostly unrelated to conserved binding modules and display diverse binding patterns that often coincide with the various serovar groups of *Listeria* [17,33]. The interaction between binding domains and ligands is presumably charge-dependent and often characterized by exceptionally high affinity [33,36] [Eichenseher F *et al*. Unpublished Data]. For CBDs of *Listeria* phage endolysins, equilibrium affinity constants in the pico- to nano-molar range have been determined, which is comparable to or exceeds the affinity of antibodies to their antigens [17,33]. The exact nature of the ligands recognized by the CBDs has been elucidated only in a few cases, including the aforementioned highly conserved domains. More recently, the CBD of *Listeria* phage endolysin PlyP35 was demonstrated by biochemical and genetic analyses to recognize *N*-acetylglucosamine residues within the listerial polyribitol-phosphate teichoic acids [72], whereas teichoic acids were not required for attachment of three other *Listeria* phage lysins (Ply118, Ply511 and PlyP40) to the cell wall [Eugster MR, Loessner MJ. Wall teichoic acids restrict access of bacteriophage endolysin Ply118, Ply511, and PlyP40 cell wall binding domains to the *Listeria monocytogenes* peptidoglycan (2012), Submitted].

As mentioned above, an EAD may possess the ability to bind to the bacterial cell surface independently of its respective CBD and contain features conferring inherent cell wall specificity. This was demonstrated for the clostridial lysin CD27L, where the deletion of the binding domain did not significantly widen the spectrum of susceptible strains and the truncated enzyme fused to GFP was able to decorate *Clostridium difficile* cell walls [73]. Some lysins have been reported to require a CBD for full lytic activity from without [74–78], while others are equally or even more active when the binding domain is deleted [73,79–81]. The term `nonclassical' lysin has been coined for the latter case [21]. It should be noted that when lytic activity is determined by assays that rely on diffusion of an enzyme through a solid matrix, such as the plate lysis and overlay assays (see below), isolated EADs often appear to have higher activity than the respective full-length lysins, possibly due to their smaller size and lower cell wall affinity [81]. The question of whether a CBD is necessary for (full) lytic activity has been suggested to depend on the net charge of the EAD, with positively charged catalytic domains tending to function independently of the presence of a binding module [21]. An explanation for cases where deletion of a CBD increases lytic activity was offered by Low *et al.* [80], who suggested that the C-terminus of the *Bacillus anthracis* prophage endolysin PlyL inhibits its catalytic domain by intramolecular interaction unless bound to a specific ligand only present in the cell wall of *Bacillus cereus*. This proposed model explains the observation that C-terminal truncation of PlyL increases activity against all tested *Bacillus* species except for *B. cereus*.

Crystal structures

While the 3D crystal structures of a handful of single-domain globular phage lysins have been published [82–86], only two complete modular endolysins have been successfully crystallized to date, despite repeated efforts to do so (reviewed in [31]). The first modular structure reported was that of the pneumococcal phage lysin Cpl-1, which features an N-terminal muramidase domain and a C-terminal CBD consisting of six consecutive tandem repeats arranged in two separate structural regions, with both domains forming a hairpin structure [87]. It has been suggested that the two functional modules interact prior to binding, and the CBD ensures correct orientation of the enzyme towards its substrate. Similar cooperative mechanisms have also been proposed for other enzymes that rely on a

CBD for full lytic activity [73]. The second modular endolysin that was crystallized was that of *Listeria* phage PSA, a zinc-dependent amidase [75]. The CBD of PlyPSA assumes an interesting fold, featuring an internal structural duplication without significant sequence relatedness. Typically, the individual domains of modular endolysins are connected by short and mostly flexible linker regions, as is apparent in both Cpl-1 and PlyPSA crystal structures. This inherent flexibility makes it difficult to crystallize complete proteins. For this reason, most of the crystal structures reported comprise only one domain [73,76,80,88,89]. Interestingly, the EAD of the zinc-dependent *C. difficile* endolysin CD27L is structurally very similar to that of PlyPSA, whereas their sequence identity is only 30% [73], which is – similar to the aforementioned internal duplication within the CBD of PlyPSA – a prime example for convergent evolution of enzymes. Besides PlyPSA and CD27L, the requirement of a divalent metal cation for lytic activity has been demonstrated for several other lysins by elucidating their crystal structures [80,88], and it has been suggested for others based on the presence of conserved zinc-binding motifs [90]. The catalytic modules of PGH can be classified into different fold types, with many of the crystallized glycosidases falling into the glycosyl hydrolase families 24 and 25 (GH-24, GH-25) [21,31]. An excellent overview of the different families and fold groups of amidases and endopeptidases is presented by Firczuk and Bochtler [91]. While x-ray crystallography is considered the gold standard and is the most commonly used method for protein structure determination, other approaches to determine or predict PGH structures have been attempted, including nuclear magnetic resonance techniques [92], homology modeling [93,94] or the combination of various biophysical methods [68].

Endolysin activity

The degradation of PG by the action of an endolysin under normal circumstances leads to lysis of the bacterium (i.e., cell death and disintegration of the major structural components of the cell). This event can be detected through different macroscopic phenomena, which are quantified in numerous PGH assays. These phenomena include: the decrease in turbidity (i.e., clearing) of a bacterial or PG suspension (spectrophotometrically measured in the turbidity reduction assay [TRA]) or alternatively, development of a clear zone within a semisolid matrix with bacteria or PG embedded at high optical density (e.g., zymogram or overlay assay); the decrease in the number of CFUs of a suspension of live bacteria determined by serial dilution plating (viable counts assay); or the absence of overnight bacterial growth in liquid (MIC assay) or agar (plate lysis assay) media. A recent review by Nelson *et al.* provides a detailed description of the various methods that have been used to measure endolysin activity [26]. Thus, this article will be limited to only brief explanations for each method. Figure 3 exemplifies some of the assays described here.

The TRA or photometric lysis assay (Figure 3A) is one of the most commonly used methods and can be performed with suspensions of live [40,42,95,96] or inactivated whole cells [14,16,97] or PG preparations [90]. After addition of a defined amount of enzyme to a turbid suspension, the decrease in optical density over time is recorded. This enables quantification of enzymatic activity by either measuring the steepest slope of the resulting lysis curve [58,75,98] or the amount of enzyme required to reduce the optical density by a certain percentage (e.g., 50%) of its initial value within a defined period of time [50,99,100].

Zymograms (Figure 3B) and overlay assays can be considered variations of the TRA, which are, however, not as well suited for quantitative analysis, and are where the clearing takes place within a semisolid matrix rather than a liquid suspension. A zymogram is an SDS-PAGE assay of an endolysin preparation where bacteria or naked PG are embedded in the gel to the point of opacity, and lytic activity of individual protein bands can be seen by the development of clear zones after renaturation of the lytic proteins by SDS removal (soaking

the gel in water or buffer). It is a very sensitive assay and can be used to demonstrate that lytic activity of a preparation is ascribed to a specific band in a parallel SDS-PAGE [101,102]. The overlay assay is used primarily for screening expression libraries (*Escherichia coli*, in most cases) to identify clones producing endolysins or other PGHs. A replica of an original transformation plate containing colonies with induced protein expression is exposed to chloroform vapor to liberate the expression library-encoded proteins. The plate is then overlaid with soft agar containing bacterial target cells, and scored for the identification of lytic protein expression by emerging clear halos that form around the positive colonies [16,79].

While the TRA, zymogram and overlay assays measure enzymatic activity *per se*, the following assays are used to quantify the antimicrobial activity of endolysins as a consequence of their enzymatic action. In the viable count assay, PGH is added to a suspension of live cells, and the number of CFUs is determined at certain time intervals by serial dilution plating, in comparison with a no-enzyme control, yielding the reduction in bacterial CFUs achieved by a known amount of enzyme, over a given time period, when subjected to a defined number of CFUs. Often, viable counts are performed in parallel to a TRA [40,103].

The MIC assay (Figure 3C) asks for the lowest concentration of an antimicrobial agent such as a PGH that can prevent overnight growth of a bacterial culture [104]. Serial dilutions of the enzyme are prepared, usually in a 96-well dish, and mixed with a defined concentration of bacteria in a suitable growth medium. After overnight incubation, the MIC can be determined by identifying the lowest enzyme concentration that yields a clear well (i.e., no visible bacterial growth) as opposed to turbid wells, reflecting a subinhibitory concentration of enzymes [58].

By contrast to the MIC assay, the plate lysis assay (or `spot-on-lawn' assay) (Figure 3D) is performed on a solid agar plate [40,102]. An exponentially growing culture of target cells is spread on the plate, and defined amounts of endolysin in small volumes (mostly 10 μl) are spotted on the air-dried plate. Overnight incubation allows for growth of a bacterial lawn, whereas antimicrobial activity of a protein is indicated by a clear lysis zone within the lawn where growth has been prevented. In analogy to the MIC assay, spotting a serial dilution of enzyme on a freshly plated bacterial lawn enables determination of the smallest amount of protein required to produce a visible lysis zone. The term `minimum inhibitory amount' has been coined for this assay [105].

It should be noted that an enzyme may yield inconsistent results (at least on a quantitative level) when tested in multiple assays [106]. This is not surprising given that different methods ask different questions (e.g., enzymatic vs antimicrobial activity), differ in sensitivity and every method may have a specific inherent bias, favoring enzymes with certain biochemical properties, such as regarding molecule size, hydrophobicity or cell wall affinity (e.g., assays using liquid media vs solid matrices). Therefore, endolysins should ideally be tested in more than one activity assay using assay parameters that mimic the intended applications as closely as possible. Bacteriophage endolysins characterized to date differ vastly in their reported lytic activities, ranging from 100 to 10^8 units/mg. However, quantitative comparison of results from different laboratories is hampered by the multitude of assay types, assay conditions and unit definitions used [90], even though efforts to standardize the determination of endolysin activity have been made [98]. An interesting approach to quantifying lytic activity was presented by Mitchell and colleagues, who developed a model based on TRA that takes into account the combined effects of the chemistry of bond cleavage and the physical mechanisms of cell lysis, and enables estimation of reaction rate constants for PGH [107]. The model has been validated using

egg-white lysozyme, a single-domain globular enzyme. However, the method can also be applied to estimate kinetics for modular enzymes. In addition, the use of model comparison methods may be necessary when trying to estimate rates for multistep reactions on cell surfaces based on turbidity data alone [Weitz J, Pers. Comm.]. Similarly, Levashov *et al.* presented a simplified model for the quantitative analysis of lysis kinetics particularly pertaining to Gram-negative bacteria [108].

Endolysins as antimicrobials

Bacterial resistance to endolysins

In light of rapidly emerging bacterial resistance to antibiotics, novel antimicrobials that are more refractory to resistance development are urgently needed. As a prominent example, the prevalence of methicillin-resistant *S. aureus* (MRSA) has dramatically increased during the past decade, turning from a once primarily nosocomial pathogen to a major cause of community-acquired infections (reviewed in [109]). The (over-)use of broad-range antibiotics (e.g., penicillin and tetracycline) accelerates the distribution of resistance genes within the bacterial community by placing selective pressure not only on the target pathogen, but also on commensal organisms [26]. In this regard, the genus- or species-specificity of most endolysins presents an important advantage over classical broad-range antibiotics. Furthermore, the coevolution of bacteriophages and their hosts is believed to have led to the endolysins binding to and cleaving highly conserved and highly immutable targets in the cell wall, presumably making the formation of resistance a rare event and thereby ensuring the phage's survival [24]. The extracellular nature of the target PG also limits the number of possible mechanisms of resistance to endolysins when applied from without, insofar as many classical resistance mechanisms target antimicrobials acting inside the cell. Examples of intracellular resistance mechanisms include the reduction of membrane permeability, active efflux of compounds from the cell and inactivation of antimicrobials by cytoplasmic enzymes [29,110]. Several studies from the Fischetti laboratory have attempted to generate and identify strains that are resistant to native or engineered phage lysins [24]. Cells of *S. pneumoniae* were repeatedly exposed to low concentrations of the Pal endolysin either on agar plates or to increasing concentrations in liquid culture, but no strains with resistant phenotypes were recovered [103]. Similar experiments were conducted with the *B. anthracis* lysin PlyG [40]. In this study, *Bacillus* cells were additionally subjected to mutagenesis with methane–sulphonic acid ethyl ester in an effort to accelerate recovery of PlyG-resistant strains. However, no such strains were found, whereas the same method yielded 1000–10,000-fold increases in novobiocin and streptomycin resistance. Similarly, attempts to create MRSA strains that were resistant to the chimeric endolysin construct ClyS were not successful, while the MIC of mupirocin against the same strain repeatedly exposed to the antibiotic increased approximately tenfold over the course of 8 days [111]. Despite these encouraging results, it should be noted that reports of bacteria that are resistant to other PGHs exist. Resistance against human lysozyme has been described and can be achieved by various secondary modifications of the cell wall, such as *O*-acetylation and *N*-deacetylation of the PG and *D*-alanylation of teichoic acids [61,112]. Similarly, several *S. aureus* strains that are resistant to the bacteriocin lysostaphin have been identified [113–115]. In most cases, this resistance could be ascribed to modifications within the pentaglycine bridge (such as reduction to a single glycine residue [114] or incorporation of a serine residue [113]), which is the target of lysostaphin [23] and also believed to be the most variable part of staphylococcal PG [52]. A theoretically plausible resistance mechanism for all PGHs applied from without is the masking of a binding ligand or PGH cut site through cell wall modifications that create sterical hindrance. However, no mechanism resembling this postulation has yet been reported for bacteriophage endolysins. With the limited data available to date, there is no definitive answer to the question of how susceptible these enzymes are to bacterial resistance strategies.

Safety & specificity

From a safety point of view, the high specificity of endolysins is among their most beneficial properties. Especially in food safety and medical applications, the fact that these enzymes specifically destroy a target pathogen without affecting the often desired commensal microflora gives them an advantage over many commonly used antibiotics or chemical preservatives. Regarding systemic administration of lysins in humans or animals, a major concern is the release of proinflammatory cellular debris associated with bacterial lysis such as teichoic acids, lipoteichoic acids and PG, which may potentially cause serious complications, such as septic shock and multiple organ failure [116]. Entenza *et al.* found an increase in proinflammatory cytokine concentrations in mice treated by continuous intravenous infusions of Cpl-1 [117], whereas Witzenrath and colleagues reported reduced cytokine concentrations compared with untreated animals when the same enzyme was administered in intervals of 12 h [118]. The difference between the two studies was explained by the possibility that continuous exposure to Cpl-1 at high concentrations results in enhanced fragmentation of the bacterial cell wall, leading to a higher level of proinflammatory molecules [25]. Finally, due to their proteinacious nature, endolysins are noncorrosive and biodegradable [26].

Immunogenicity

When considering systemic or mucosal applications of endolysins in humans or animals, the question of a potential immune response needs to be addressed. Given their proteinacious nature, it is not surprising that antibodies against these enzymes can be raised, as demonstrated in several studies. When rabbits where immunized with the pneumococcal phage lysin Cpl-1, the resulting hyperimmune serum slightly decreased or slowed the *in vitro* efficacy of Cpl-1 against pneumococci without completely inactivating the enzyme [100]. In addition, mice that were injected with several doses of Cpl-1 in the same study tested positive for IgG against the phage lysin. However, when immunized and naive mice in comparison were challenged with *S. pneumoniae* intravenously and treated with Cpl-1, there was no significant difference between the two groups regarding reduction of bacterial numbers by the enzyme, suggesting that the produced antibodies are not able to inhibit the lysin *in vivo*. Several other studies using different endolysins and different pathogens (*Streptococcus pyogenes*, *B. anthracis* and *S. aureus*) also reported that antibodies against the lysins could be raised [25], but no adverse side effects or anaphylaxis was observed after endolysin administration in animal infection models, and the antibodies were not able to inactivate the enzymes [119,120]. An explanation offered for these findings is that the high binding affinity reported for some endolysin CBDs [17, 33] [Eichenseher F *et al.* Unpublished Data] and the fast kinetics of these enzymes outperform the host's immune response [25,119].

The half-life of Cpl-1 when administered systemically in mice has been shown to be approximately 20 min, which is comparable to other proteins [100]. Therefore, lysins would need to be delivered repeatedly or by intravenous infusion if a sufficient systemic concentration during the course of the treatment was to be maintained [25]. Alternatively, modification of endolysins in order to extend their half-life is theoretically possible and has been attempted recently. PEGylation of biological molecules in order to decelerate their systemic clearance is a strategy that has been applied successfully in the past [121–123]. However, a recent study reported that cystein-specific PEGylation of Cpl-1 abolished its antibacterial activity, suggesting that this strategy is not suited for this type of modular PGH [124]. By contrast, dimerization of Cpl-1 through introduction of C-terminal cystein residues and subsequent formation of disulfide bonds not only increased the enzyme's specific activity against pneumococci, but also showed a tenfold deceleration of plasma clearance.

As natural dimerization has been suggested for several PGHs, this strategy may also proove successful for other modular lysins [125].

Synergy

Synergistic effects between two antimicrobial agents can significantly reduce the doses required for certain applications and increase the efficacy of a treatment, while at the same time potentially reducing the risk of resistance development by attacking two different targets simultaneously. Synergy has been demonstrated for a multitude of combinations between PGH or PGH and other classes of antimicrobials. A synergistic effect between two lysins with different cut sites in the PG can be explained by the enhanced destructive effect when cleaving two different bonds at the same time within a 3D network. Alternatively, the cleavage of the first bond by one enzyme could result in better accessibility to the second target site by the other lysin, causing a faster degradation of the substrate [105,126]. Examples for synergy between two PGHs include the combination of the pneumococcal phage lysins Cpl-1 and Pal [126], and the combination of the staphylococcal phage lysin LysK and lysostaphin [127]. In both cases, the more-than-additive effect was demonstrated *in vitro* by a classical checkerboard assay. For Cpl-1 and Pal, the synergistic action was additionally shown *in vivo*, using a pneumococcal sepsis mouse model [119]. Similarly, the chimeric streptococcal/staphylococcal phage lysins λSA2-E-Lyso-SH3b and λSA2-E-Lyso-SH3b [42] were shown to act synergistically with lysostaphin *in vitro* and in a mouse model of bovine mastitis [105]. Cpl-1 also exhibited synergy in combination with the antibiotics penicillin and gentamicin [128], similar to the staphylococcal phage lysin MV-L, which killed vancomycin-intermediate *S. aureus* in synergistic action with glycopeptide antibiotics [120], and the aforementioned chimeric staphylococcal lysin ClyS, which was found to be capable of curing mice from MRSA-induced septicemia in a synergistic fashion with oxacillin [99]. Furthermore, there are various recent examples of synergistic effects between phage endolysins and other antimicrobial agents, including the staphylococcal phage lysin LysH5, which reduces *S. aureus* numbers in cows' milk in synergy with the bacteriocin nisin [129], and also exhibits synergy with the virion-associated PGH HydH5 from *S. aureus* phage vB_SauS-ΦIPLA88 [130], and the *Listeria* phage endolysin PlyLM, which was shown to significantly reduce monolayers of *Listeria monocytogenes* in combination with proteinase K [131].

Engineering of endolysins: tailor-made antimicrobials

Endolysins have been optimized through evolution to cause fast and efficient lysis of the host cell from within, thereby ensuring the phage's survival. However, this does not preclude that there is still potential for improvement when it comes to application from without, especially in complex environments such as certain food matrices, blood or on mucous membranes. Protein engineering strategies such as domain swapping or random mutagenesis can alter binding and lytic properties of PGHs and thereby potentially optimize these proteins for specific applications. This section gives an overview of the recent advances in molecular engineering of endolysins. Table 1 summarizes various types of modifications and their effects on protein properties.

Domain recombination: rational design

The modular architecture of phage endolysins and the knowledge we have gained from available crystal structures and bioinformatics (e.g., regarding sequence relatedness between individual domains and the location of linker regions) make it possible to design and create functional protein chimeras consisting of multiple modules of unique origin [17]. There is evidence that each domain within such heterologous fusion constructs retains its parental function [102], suggesting that rational design and modular engineering enable the creation

of novel tools with predicted and desired properties for detection and control of pathogens. There also exist seemingly natural chimeras such as the *Listeria* phage endolysin PlyPSA [132] or the pneumococcal phage Dp-1 endolysin Pal [133], indicating that horizontal gene transfer by recombination-driven interchange of endolysin modules occurs naturally within phage populations or between phage and bacterial hosts, as has been reported previously [134]. PlyPSA features a CBD that is highly similar to those of other *Listeria* phage lysins, whereas its EAD is related to amidase domains from *Bacillus* and *Clostridium* phages. These findings corroborate the idea of creating tailor-made antimicrobials and detection tools by harnessing these basic evolutionary principles. The earliest approaches to altering properties of PGH by domain shuffling date back to the early 1990s, when Diaz *et al.* laid the groundwork for modular engineering of endolysins by demonstrating that exchange of functional domains between the pneumococcal autolysin LytA and the phage lysin Cpl-1 switches catalytic activity and regulatory properties of the resulting chimeric enzymes [135]. The same group was able to swap cell wall specificity of the choline-dependent lysozyme LytA by exchanging its choline binding domain (ChBD) with the choline-independent CBD of the amidase endolysin Cpl-7 and *vice versa* [67]. This concept was extended beyond the genus border by Croux *et al.*, who created a fusion construct consisting of the EAD of the *Clostridium acetobutylicum* autolysin Lyc and the ChBD of Cpl-1, thereby rendering the chimera active against choline-containing pneumococcal cell walls and abolishing its activity against clostridial cell walls [136]. In a reverse approach, fusing the Lyc CBD to the EAD of LytA increased activity against clostridial cell walls 250-fold [137]. Similar results with inter-generic fusions were reported by Sheehan *et al.*, who combined an EAD from the lactococcal phage Tuc2009 with the ChBD of LytA [138], and more recently by Donovan *et al.*, who fused full-length and truncated versions of the *Streptococcus agalactiae* phage B30 endolysin to mature lysostaphin, yielding enzymes that were active against both streptococcal and staphylococcal cells [102]. The same laboratory created the aforementioned fusions (λSA2-E-LysK-SH3b and λSA2-E-Lyso-SH3b) of the endopeptidase of streptococcal phage λSA2 lysin with the *Staphylococcus*-specific SH3b CBDs of either the endolysin LysK or lysostaphin, in which the exchange of CBDs bestowed activity against staphylococci on the streptococcal enzyme, while still maintaining considerable streptolytic activity [42]. Streptococci and staphylococci have been reported to account for more than 75% of cases of bovine mastitis, a disease with tremendous economic impact on agriculture worldwide [139], for which reason agents that specifically kill bacteria from both genera are of high interest.

An elegant way to visualize cell wall specificity of CBDs and to evaluate the effects of combining multiple CBDs on the binding properties of the resulting constructs is to fuse the binding modules to GFP or other fluorescent markers and detect cells that are recognized by the proteins by fluorescent microscopy (Figure 4A) [17,33,69,140–142]. Using this method, our laboratory was able to demonstrate that combining the CBD of *Listeria* phage endolysin Ply500 (CBD500), which is specific for serovar 4, 5 and 6 strains, with that of PlyP35, which recognizes the majority of serovar 1/2 and 3 strains, resulted in heterologous GFP fusion constructs that are able to recognize and brightly decorate a broad spectrum of *Listeria* cells from all species and serovars [140]. This is a property that is highly desirable when developing methods for detection of *Listeria* cells, which include human and animal pathogens, such as in food samples (see below). The same is true for high binding affinity, which could also be achieved by modular engineering in the same study. By duplication of CBD500, a 50-fold increase in affinity to the cell wall was achieved ($K_A = 2.93 \times 10^{10}$ M^{-1}), enabling highly efficient immobilization of target cells. New results from the laboratory with staphylococcal lysins suggest that binding affinity can be further potentiated with increasing copies of the same CBD in one fusion construct [Eichenseher F *et al.* Unpublished Data]. Furthermore, enhanced cell wall affinity presumably causes tighter binding at high ionic strength, which explains the observation that the Ply500 endolysin with a duplicated

CBD exhibited enhanced activity at elevated salt concentrations. Similar to the studies with protein chimeras of pneumococcal and clostridial origin, exchange of *Listeria* phage CBDs of different serovar specificity also yielded enzymes with swapped lytic properties and enhanced activity, which constitute interesting antimicrobial candidates for control of the pathogen [140]. In certain cases, modular engineering of endolysins may also solve solubility problems, thereby ensuring efficient production and purification of otherwise insoluble lytic proteins. This was demonstrated by Manoharadas *et al.*, who created a fusion of the insoluble *S. aureus* phage P68 endolysin with a minor coat protein of the same phage [143]. The protein was readily purified and exhibited antimicrobial activity against *S. aureus*. Another study attempted to reduce the probability of resistant strain development by replacing the native SH3b domain of the *S. aureus* phage Twort endolysin [144] with the unrelated ΦNM3 binding domain, creating the ClyS chimera [99]. The authors argued that SH3b domains promote resistance formation by recognizing the easily mutable pentaglycine crossbridge of staphylococcal PG, whereas the NM3 CBD binds to a different yet unknown ligand. As described above, repeated exposure to ClyS did not yield any resistant strains. However, the study lacks a head-to-head comparison of the chimeric construct with the parental SH3b-containing Twort endolysin. An alternative approach to tackle the resistance problem is pursued by the Donovan laboratory, which has created fusion proteins harboring three functional EADs with different catalytic specificities (CHAP, M23 endopeptidase and amidase) that are highly active against staphylococcal target cells. The simultaneous attack on three different bonds within the PG is expected to further reduce the chance of resistant strain development [29] [Becker SC *et al.*, Unpublished Data].

Random mutagenesis directed evolution

As opposed to domain shuffling, where complete functional modules are reassembled and which mimics the naturally occurring horizontal gene transfer among phages and their hosts, protein evolution can also be directed artificially by inserting point mutations into a parental nucleotide sequence. In cases where the effects of replacing certain amino acids on protein conformation and function can be predicted, this can be achieved by site-directed mutagenesis. An alternative approach is random mutagenesis of protein coding sequences followed by screening of the resulting expression library for clones with desired properties. A prime example for improving lytic activity of a phage endolysin by random mutagenesis was presented by Cheng and Fischetti [79]. In this work, the gene coding for the *S. agalactiae* phage endolysin PlyGBS was subjected to two different methods of DNA mutagenesis, using an *E. coli* mutator strain or an error-prone PCR approach. In both cases, an expression library of PlyGBS mutants was screened by a soft agar overlay assay, where clones with increased lytic activity were selected by visual evaluation of the lysis zones. After repeated rounds of mutagenesis and screening, a mutant with 28-fold activity compared with the parental enzyme was identified. It featured a frameshift mutation resulting in C-terminal truncation, leaving only the endopeptidase domain of the enzyme intact, with an additional 13 `out-of-frame' amino acids at the C-terminus. It should be noted that the choice of the screening method in this study may select for enzyme mutants with high diffusion capability such as the C-terminal truncation, which is a comparably small molecule lacking a CBD. This general method can potentially be applied for identification of endolysin mutants with enhanced activity under various conditions, such as under high salt or low pH conditions or in certain food products. Whereas the mutagenesis follows well-established protocols, the screening method has to be adapted for every individual condition.

There are also examples in the literature describing the use of site-directed mutagenesis for deliberately changing the properties of PGH. Low *et al.* were able to show that a positive net charge is favorable for an EAD to function independently of its CBD, and consequently CBD dependence can be eliminated by increasing the positive charge on the EAD surface

through amino acid replacement [21]. This may be relevant in cases where efficacy of an enzyme is limited by its size, such as when targeting *B. anthracis*, whose S-layer acts as a molecular sieve. Mayer and colleagues described another way of fine-tuning enzymatic properties when demonstrating that a mutant enzyme featuring a point mutation that did not affect the catalytic center exhibited differences in species specificity compared with the parental protein [73].

Fusion to cationic peptides & protein transduction domains

The bulk of research on phage endolysins as potential antimicrobials to date has focused on Gram-positive bacteria, whereas the chances of success when targeting Gram-negative bacteria have been considered low, due to the OM effectively shielding these organisms from the outside. However, recent developments in the field of OM permeabilizers (OMPs) [145] have raised hopes of expanding the arsenal of endolysin-based antimicrobials to tackle important Gram-negative pathogens such as *Pseudomonas*, *E. coli* and *Salmonella* (reviewed in [26]). OMPs can be classified into two groups according to their mechanism of action [146]: chelators such as EDTA and certain organic acids, which bind to and remove OM-stabilizing divalent cations from their binding site, thereby disrupting the membrane; and polycationic agents (including polycationic peptides), which competitively displace the cations, resulting in OM disorganization. A recent study describes the use of the *P. aeruginosa* phage endolysin EL188 in combination with 10 mM EDTA against antibiotic-resistant *Pseudomonas* strains. EDTA significantly increased the efficacy of the lysin; however, its therapeutic use is limited to topical application due to its blood coagulating properties [146]. Another approach presented in two recent patents [201,202] focuses on fusing PGH to various cationic, polycationic or other membrane-disrupting peptides (including natural and synthetic peptides) by molecular engineering in order to increase the efficacy of these enzymes against Gram-negative bacteria from without. Lu *et al.* reported a construct consisting of human lysozyme fused to the antimicrobial peptide cecropin, which is believed to form pores in the OM, and this rendered the PGH active against several Gram-negative species [147]. Similar strategies are theoretically possible for phage endolysins. A number of parental phage lysins have been reported to feature intrinsic positively charged and/or hydrophobic regions that supposedly interact with and permeabilize bacterial OMs. Examples include the *Bacillus amyloliquefaciens* phage endolysin Lys1521, which exhibits activity not only against Gram-positive bacteria, but also viable cells of *E. coli* and *P. aeruginosa* [148–150], and the recently described *Acinetobacter baumannii* phage endolysin LysAB2, which has a broad activity range including Gram-positive and Gram-negative bacteria such as *A. baumannii*, *E. coli*, *Citrobacter freundii*, *Salmonella enterica* and *Enterobacter aerogenes* [151]. It should be noted that from a therapeutic point of view, the use of lysins with OM-disrupting properties or of fusion constructs with peptide OMPs poses a certain risk, especially regarding the possibility of these antimicrobial peptides also interacting with eukaryotic cell membranes. This has to be empirically determined for every individual construct [26].

On the other hand, one newly proposed strategy particularly relies on the interaction of similar peptides with eukaryotic membranes: protein transduction domains (PTDs), also termed cell-penetrating peptides, membrane-permeable peptides or Trojan horse peptides, are mostly positively charged or amphipathic regions of proteins that facilitate their uptake by eukaryotic target cells [152–154]. They have been used for internalization of numerous bioactive macromolecules and have therefore been suggested as fusion tags for PGH for the purpose of directing these lytic enzymes against bacterial pathogens that are able to invade, persist and replicate inside eukaryotic cells [155]. These include *S. aureus* [156], *L. monocytogenes* [157], *S. pyogenes* [158] and *B. anthracis* [159]. New results from the Donovan laboratory indicate that a variety of PTDs fused to lysostaphin as well as parental

and chimeric staphylococcal phage lysins are capable of reducing intracellular numbers of *S. aureus* (both methicillin-sensitive *S. aureus* and MRSA strains) in bovine mammary gland epithelial cells, murine osteoblasts and human brain microvasculature epithelial cells [Becker SC et al. Unpublished Data]. The use of PTDs to target PGH against intracellular pathogens is a promising strategy. However, the technology is still in its early stages, and more experiments are needed to evaluate its therapeutic potential.

Applications of parental & engineered endolysins

Medicine

Since the discovery of lysis from without caused by bacteriophage endolysins, these enzymes have been potential candidate therapeutics for the treatment of bacterial infections of humans and animals. However, only in the face of a soaring resistance problem with classical antibiotics have researchers started focusing on medical applications of purified endolysins as alternatives to conventional treatment. So it was not until 2001 that Nelson *et al.* demonstrated the potential of the streptococcal phage lysin PlyC (then termed C1 lysin) to prevent and treat upper respiratory colonization in mice by group A streptococci [50]. Administration of a single dose of lysin to the oral cavity before inoculation with bacteria reduced the percentage of colonized mice from >70% in the control group to <30%. When already colonized animals were treated with 500 U of PlyC, no remaining bacteria were detected after 2 h. The same group reported elimination of nasopharyngeal colonization of mice by *S. pneumoniae* by a single dose of the enzyme Pal within 5 h, without effect on commensal organisms [103], and a significant reduction in vaginal and oropharyngeal colonization with group B streptococci by PlyGBS [160]. Not only colonization of mucous membranes, but also systemic bacterial infections have been successfully treated with endolysins. When mice were infected intravenously with *S. pneumoniae*, a 2-mg dose of Cpl-1 given 1 h after the bacterial challenge reduced pneumococcal titers in the blood from 4.7 log CFU/ml to undetectable levels, with 100% of the mice surviving after the treatment (compared with 20% in the control) [100]. Both Cpl-1 and Pal were able to protect mice from pneumococcal bacteremia induced by intraperitoneal (ip.) injection. When used in combination, the enzymes eliminated the infection at significantly lower doses (2.5 μg for each enzyme compared with 200 μg in the individual treatment), exhibiting a synergistic effect *in vivo* [119]. More recent studies with Cpl-1 include: the treatment of *S. pneumoniae*-induced endocarditis in rats by continuous endolysin infusion, resulting in a significant reduction of aortic vegetations and CFUs in the bloodstream [117]; the prevention of otitis media as a secondary problem upon influenza infection by previous decolonization with Cpl-1 [161]; the treatment of pneumococcal meningitis in rats by either intracisternal or ip. injection of the lysin [162]; and the treatment of pneumonia with Cpl-1 [118]. In the latter study, 100% survival of mice suffering from otherwise fatal pneumonia was achieved when the lysin was injected ip. 24 h after transnasal infection, compared with 86% with amoxicillin. These results demonstrate the capability of the endolysin of curing an established infection [25]. When treated after onset of bacteremia (48 h after infection), Cpl-1 and the antibiotic saved 42 and 71% of the mice, respectively.

Besides treatment of streptococcal infections, remarkable results were achieved with the PlyG endolysin from the γ-phage of *B. anthracis*, an agent relevant in connection with biowarfare. Mice infected ip. with *Bacillus* cells and treated with buffer died after 2 h, whereas 70–80% of PlyG-treated mice survived until the end of the experiment at 72 h. Furthermore, PlyG was able to eliminate *Bacillus* spores in combination with a germinating agent [40]. Another *Bacillus* phage lysin, PlyPH, which features an exceptionally broad pH range of activity (pH 4–10.5), was able to protect 40% of mice from death after ip. infection of *Bacillus* cells [41].

There have been several reports on controlling staphylococcal infections (including MRSA) in animal models within the past 5 years. The first study from 2007 used the MV-L endolysin from phage ΦMR11 to eliminate MRSA from murine nares. When the same enzyme was injected ip. into mice 0, 30 and 60 min after systemic infection with MRSA, 100, 100 and 60% of the animals survived the challenge, respectively [120]. In addition, the chimeric lysin ClyS significantly reduced MRSA nasal colonization in mice (2 log reduction after one dose), eradicated skin infections more efficiently than the standard topical antimicrobial mupirocin (3 log vs 2 log) and protected ip.-infected animals in a murine septicemia model from death, individually and synergistically with oxacillin [99,111]. Successful nasal decolonization was also reported for the truncated phage lysin LysK (CHAPk) [163], and both GH15 and P-27/HP endolysins were reported to considerably reduce bacterial numbers in murine spleens and/or protect systemically infected animals from death [164–166].

Food safety

Numerous studies have demonstrated the potential of bacteriophage endolysins and other PGHs for the control and detection of food-borne pathogens. Again, a big advantage of lysins over other antimicrobial agents is their high specificity for the target pathogen, leaving the natural and often desired bacterial flora of the food product untouched. A review article by Callewaert *et al.* summarizes the most recent developments in this field [167]. One obvious control strategy is the addition of purified endolysins to food products as biopreservatives. Obeso and colleagues showed that the staphylococcal phage endolysin LysH5 rapidly kills *S. aureus* in pasteurized milk, reducing bacterial numbers below the detection level within 4 h [46], and that it acts synergistically with the bacteriocin nisin [129]. In addition, the streptococcal lysins B30 [102] and Ply700 [141], the chimeric streptococcal–staphylococcal constructs λSA2E-Lyso-SH3b and λSA2E-LysK-SH3b [105] and the *Clostridium butyricum* phage ΦCTP1 lysin [168] have been reported to be active in milk or milk products. An interesting discovery is the high thermoresistance of three *Listeria* phage endolysins Ply118, Ply511 and PlyP35, the latter retaining considerable lytic activity after heating to 90°C for 30 min. These thermostable enzymes may find application in food products that undergo heat treatment such as pasteurized milk products [90]. Ply511, Ply118 and Ply500 also showed promise as antilisterial agents on other food products such as iceberg lettuce, where reduction of viable counts of *L. monocytogenes* by up to 2.4 log units after storage for 6 days was achieved [Guenther *et al.*, Unpublished Data]. Another example of antimicrobial efficacy of phage lysins on the surface of solid foods is the inhibition of *Erwinia amylovora* growth on pears by soaking the fruits in lysates of *E. coli* cells producing an *Erwinia* phage lysozyme [169]. An alternative approach for the control of food-borne pathogens by endolysins is their production and secretion or release by starter organisms used in fermentation processes, such as *Lactococcus lactis*. This has been reported for endolysins from *Listeria* [81,170,171] and *Clostridium* phages [172], as well as lysostaphin [170], even though application in a food product has yet to be demonstrated.

Substantial progress has been made in the detection of food-borne pathogens using high-affinity CBDs as alternatives to standard detection methods. Kretzer *et al.* showed that in foods naturally and artificially contaminated with *L. monocytogenes*, detection rates of >90% could be reached by CBD-based magnetic separation, using paramagnetic beads coated with *Listeria* phage CBDs (Figure 4B) [173]. This method was demonstrated to be superior to the standard plating procedure in terms of sensitivity and time requirement, and also to be applicable for the detection of other pathogens such as *B. cereus* and *Clostridium perfringens*. In addition, the possibility of coupling this highly sensitive technique with quantification by real-time PCR has been demonstrated [174]. The fusion of CBDs to fluorescent markers such as GFP, CFP or dsRed not only allows for the elucidation of

binding properties, but also creates tools for the specific detection and differentiation of target cells (Figure 4A). Coupling eight different *Listeria* phage CBDs with different cell wall specificity to various differently colored reporter proteins created a set of tools for microscopic detection and differentiation of *Listeria* cells with serovarto strain specificity [17]. This method enabled the distinction of various *L. monocytogenes* strains following recovery from contaminated food by CBD magnetic separation. CBD-based detection methods have also been reported for *B. anthracis* [175,176]. In the latter study, a ten-amino acid synthetic peptide from the C-terminal region of PlyG was shown to be sufficient for strain-specific detection of *B. anthracis* surrogates when coupled to fluorescent Qdot® nanocrystals (Invitrogen, MD, USA).

Agriculture

Besides their application as purified proteins to control pathogens on food products, endolysins can also be expressed by transgenic plants to prevent infection by phytopathogenic bacteria. As a prominent example, Düring *et al.* produced transgenic potatoes expressing T4 lysozyme, which were resistant against the plant pathogen *Erwinia carotovora*, which causes soft rot [177,178]. The enzyme accumulates in the plant tissue and comes into contact with the bacteria after destruction of the plant cells by bacterial pectinases. To take this concept one step further, plants may also be used as bioreactors, not for their own protection, but for the inexpensive and large-scale production of antimicrobial proteins to be used in human and veterinary medicine. This was demonstrated by Oey and colleagues, who produced the *S. pneumoniae* phage endolysins Cpl-1 and Pal and the group B streptococcal lysin PlyGBS in the chloroplasts of tobacco plants at levels >70% of the plants' total soluble protein [179,180].

Furthermore, in animal agriculture, PGHs have been used to prevent or treat diseases and reduce the risk of transmitting pathogens into food. Bovine mastitis, an inflammation of the mammary gland in cows, is the most widespread disease in cattle and causes annual losses of up to US$2 billion in the USA alone [181]. As antibiotic therapy is often ineffective [182] and contributes to resistance development, alternative antimicrobials are urgently needed. Staphylococci are among the most prevalent mastitis-causing pathogens, and therefore lysostaphin was the first PGH that was intensely studied for its potential to prevent and treat mastitis. It has been demonstrated to be effective against intramammary *S. aureus* infection when infused into the teat canal of infected animals, both in a mouse model [183] and in cows [184]. Furthermore, transgenic production of the bacteriocin in mice [185] and in cows [186] rendered the animals resistant to *S. aureus*-induced mastitis. However, due to the existing resistance problem (see `Bacterial resistance to endolysins' section), the use of a combination of antimicrobials might be a safer strategy to avoid new resistant-strain development. To this end, the combination of lysostaphin and the chimeric phage lysin λSA2E-LysK-SH3b was tested against mastitis strains, and the enzymes were found to synergistically kill *S. aureus in vitro* and in a mouse model of bovine mastitis [105].

Another application of phage lysins was presented by Hoopes *et al.*, who assessed the efficacy of PlyC as a narrow-spectrum disinfectant to eradicate *Streptococcus equi*, the causative agent of equine strangles, on horse stable surfaces and equipment. In this study, the endolysin was found to be 1000-fold more active than a conventional disinfecting agent used as a control [187].

Other applications

A multitude of other applications of endolysins have been described. In recent years, the body of literature dealing with the elimination of bacterial biofilms using PGH has increased considerably. According to the NIH, approximately 80% of bacterial infections in humans

involve biofilms [26], and they are also a problem in food production and processing and various other industries [188]. Their high level of antimicrobial resistance makes biofilms difficult to eradicate (reviewed in [26]). However, recent successes in studies with endolysins hold promise for future applications. These include: the removal of static *S. aureus* biofilms by the staphylococcal phage lysins Φ11 [78] and SAP-2 [189]; the destruction of *S. pneumoniae, Streptococcus pseudopneumoniae* and *Streptococcus oralis* biofilms by various pneumococcal phage lysins, with Cpl-1 acting synergistically with the autolysin LytA [190]; the eradication of *Streptococcus suis* biofilms by the phage lysin LySMP, alone and in synergistic action with antibiotics [191]; and the lysis of *L. monocytogenes* monolayers by the amidase PlyLM in conjunction with a protease [131]. Furthermore, phage lysins can be used in biotechnology for the recovery of nucleic acids or proteins from Gram-positive cells [95], for controlled cell lysis to produce empty `ghost' cells as vaccines [192,193], for surface display of heterologous proteins on lactic acid bacteria using CBDs as anchor molecules, which can be useful for the development of live vaccine delivery systems, whole-cell absorbents and biocatalysts [194], and for the development of autoinducible *E. coli* lysis systems facilitating the release of recombinantly produced proteins [195].

Future perspective

The number of publications describing isolation and characterization of new phage endolysins, as well as potential applications of these enzymes in the fields of medicine, food safety, agriculture and biotechnology, has increased considerably during the past 5 years. Particularly with respect to the vastly increasing threat imposed by multiresistant `super bugs', this trend is expected to continue as the search for novel antimicrobials, including those based on PGH, will further intensify in the near future. From a medical perspective, phage lysins hold promise especially as topically applied therapeutics, thereby evading or reducing many potential risks associated with systemic administration, such as an adverse immune response to either the protein itself or to the release of proinflammatory bacterial cell antigens. However, recent studies involving animal models and offering answers to some of these questions raise the prospect of phage lysins becoming suitable alternatives for antibiotics in long-term systemic therapy as well. Moreover, after the first promising results with OMPs and PTDs, research focusing on endolysin-based antimicrobials for the control of Gram-negative and intracellular pathogens is expected to gain momentum in the coming years, and the dogma of endolysins being effective only against Gram-positive bacteria when applied externally is about to fall. Regarding applications in food, current regulations hamper the use of recombinantly produced phage lysins as antimicrobial food additives. However, the approval by the US FDA for the use of *Listeria* bacteriophages on certain food products in combination with promising results demonstrating efficacy of PGH in food may provide new impetus for further regulatory restrictions to be eased in the near future. Particularly in the food industry, synergistic effects between different antibacterial substances could be harnessed to provide an effective hurdle strategy against food-borne pathogens on certain products. The list of available antimicrobial agents and processes is long, and phage endolysins may become an integral part of this strategy. Finally, the molecular engineering techniques described here open up the possibility to create tailor-made PGH-based antimicrobials optimized for any intended field of application. The fast evolution of sequencing techniques will ensure identification of useful domains at an increasing pace and decreasing expense, and the use of alternative expression systems such as plants or yeast will further reduce costs of production. With an estimated 10^{31} phages worldwide, the reservoir of functional domains that can be used to create powerful tools for the detection and control of pathogens is virtually unlimited.

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Executive summary

Endolysin structure & mechanism of action

- Endolysins from a Gram-positive background feature a modular organization in which cell wall binding and catalytic activity are separated, whereas Gram-negative lysins are mostly single-domain globular proteins.
- Modular architectures and orientations of the enzymatically active domains (EADs) and cell wall binding domains (CBDs) of endolysins are manifold.
- EADs can be classified by the bond they cleave within the bacterial peptidoglycan, which can be determined by either classical biochemical assays or N-terminal sequencing or mass spectrometric analysis of digestion products.
- CBDs specifically recognize and bind to ligand molecules in the cell wall, thereby impacting the activity range of an endolysin. Some lytic domains require a CBD for full activity, while others are equally or more active without a binding module.
- 3D crystal structures of two complete modular endolysins, Cpl-1 and PlyPSA, are available and feature flexible linker regions connecting their functional domains.

Endolysin activity

- Endolysin activity can be determined by various assays relying on different macroscopic phenomena. Enzymatic and antimicrobial assays can be distinguished.
- Different assays may yield different results regarding the lytic activity of endolysins. Standardized methods are needed to allow comparison of results between different laboratories.

Endolysins as antimicrobials

- Due to their specificity and immutable targets, endolysins are expected (and reported) to be highly refractory to resistance development.
- Antibodies against endolysins can be raised, and increase in proinflammatory cytokines upon systemic application is possible. However, neither inactivation nor adverse side effects of the proteins have been observed *in vivo*.
- Numerous synergistic benefits between endolysins or endolysins with other antimicrobial agents have been described, which may be harnessed to increase efficacy of treatments and further reduce the risk of resistant strain development.

Engineering of endolysins

- The modular design of endolysins allows truncation of multidomain proteins, enhancing the ability to discern unique EAD and CBD functions.
- Creation of chimeric proteins by rearrangement of functional domains has been demonstrated for lysins from multiple species. Molecular engineering can increase lytic activity, broaden specificity, improve binding affinity, enhance solubility and reduce the chance of resistance formation, thereby optimizing lysins for specific applications.

- Random and site-directed mutagenesis of endolysin sequences can modulate physicochemical properties and enhance enzyme efficacy under desired conditions.
- Fusion to outer membrane permeabilizing peptides and protein transduction domains can render endolysins active against Gram-negative and intracellular pathogens.

Applications of parental & engineered endolysins

- Endolysins have been used successfully in various medical applications *in vivo*, including decolonization of mucous membranes and treatment of systemic infections and controlling pathogenic bacteria from various genera such as *Streptococcus*, *Bacillus* and *Staphylococcus*.
- In food applications, endolysins show promise as antimicrobial food additives when directly added to food products or secreted by lactic acid bacteria used in fermentation processes. CBDs can be utilized for highly sensitive and specific pathogen detection methods.
- Agricultural applications of endolysins include the creation of transgenic plants that are resistant to phytopathogens and the prevention and treatment of bovine mastitis.
- Various endolysins have been demonstrated to reduce or eradicate bacterial biofilms, which are a problem in human infections, food production and other industries, and feature a high level of antibiotic tolerance.

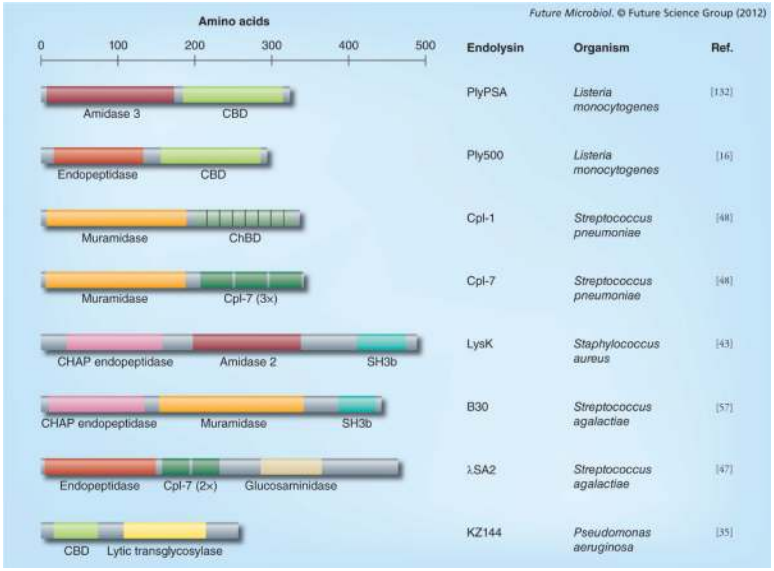

Figure 1. Schematic representation of selected bacteriophage endolysins, illustrating their modular architecture

The scale bar indicates the number of amino acids.

CBD: Cell wall binding domain; ChBD: Choline binding domain; Cpl-7: Cpl-7-like cell wall binding domain; SH3b: Bacterial Src homology 3 domain.

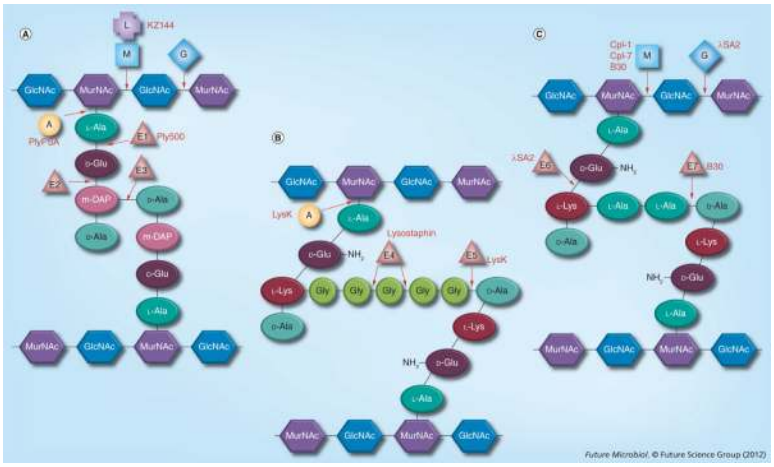

Figure 2. Structures of bacterial peptidoglycan types and cut sites of different peptidoglycan hydrolases

(A) A1γ type featuring a direct cross-link between m-DAP and D-Ala, as present in *Listeria, Bacillus* and most Gram-negative species. **(B)** *Staphylococcus aureus* peptidoglycan of the A3α type featuring a pentaglycine cross-bridge. **(C)** Streptococcal peptidoglycan (A3α) with an L-Ala–L-Ala interpeptide bridge. Sugar units are depicted as hexagons and amino acids as ovals. The different classes of peptidoglycan hydrolases are represented by different shapes and letters and their cut sites are indicated by arrows. Specific examples including the endolysins shown in Figure 1 and the bacteriocin lysostaphin are given next to the respective symbols. All five classes of enzymes are present in **(A)**, whereas only selected examples are shown in **(B & C)**. Triangles with E numbers in them represent different types of endopeptidases.

A: *N*-acetylmuramoyl-L-alanine amidase; E1: L-alanoyl-D-glutamate endopeptidase; E2: D-glutamyl-m-DAP endopeptidase; E3: Interpeptide bridge-specific endopeptidase; E4: Glycylglycine endopeptidase; E5: D-alanoyl-glycine endopeptidase; E6: D-glutamyl-L-lysine endopeptidase; E7: D?-alanoyl-L-alanine endopeptidase; G: *N*-acetyl-β-D-glucosaminidase; L: Lytic transglycosylase; M: *N*-acetyl-β-D-muramidase.

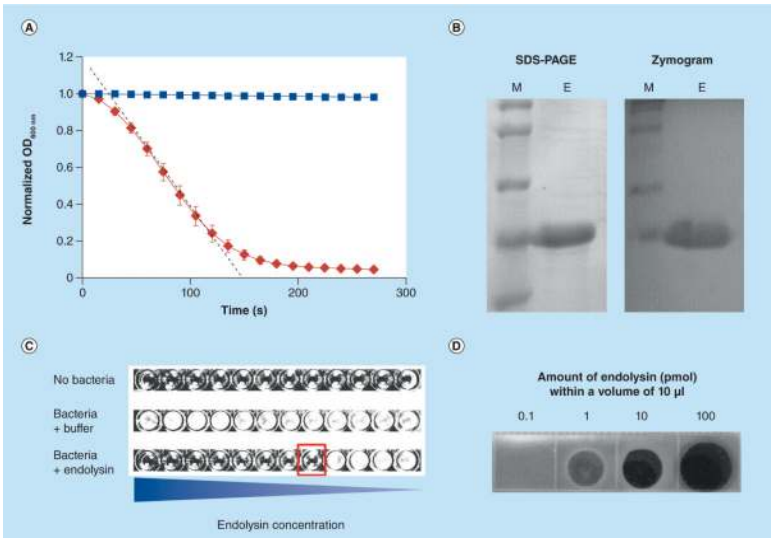

Figure 3. Assays used to determine enzymatic and antimicrobial activity of endolysins

(A) Turbidity reduction assay. The change in optical density of a bacterial suspension upon addition of buffer as control (squares) or a defined amount of endolysin specific for the target cells (diamonds) is shown. The dashed line indicates the steepest slope of the resulting lysis curve [Schmelcher M, Unpublished Data]. **(B)** SDS-PAGE and zymogram. An SDS-PAGE assay of a protein preparation containing the target endolysin at an estimated purity >90% (prominent band) and multiple contaminant proteins (faint bands) is shown on the left, and a zymogram of the same preparation with substrate cells embedded in the gel is shown on the right. Lytic activity of the endolysin is indicated by a single cleared band [Roach D, Unpublished Data]. **(C)** Determination of the MIC. A twofold dilution series of an endolysin was mixed with target bacteria in growth medium and incubated overnight (bottom row). Clear wells (dark) indicate growth inhibition, with the last clear well representing the enzyme's MIC (boxed). Control rows containing no bacteria (top) or buffer without endolysin mixed with bacteria (middle) are also shown [Schmelcher M, Unpublished Data]. **(D)** Spot-on-lawn assay. Tenfold serial dilutions of a peptidoglycan hydrolase were spotted on a freshly plated lawn of exponentially growing target bacteria (10 µl per spot). After overnight incubation, cleared spots indicate lytic activity [Schmelcher M, Unpublished Data].

E: Endolysin preparation; M: Prestained molecular weight marker; $OD_{600\ nm}$: Optical density measured at a wavelength of 600 nm.

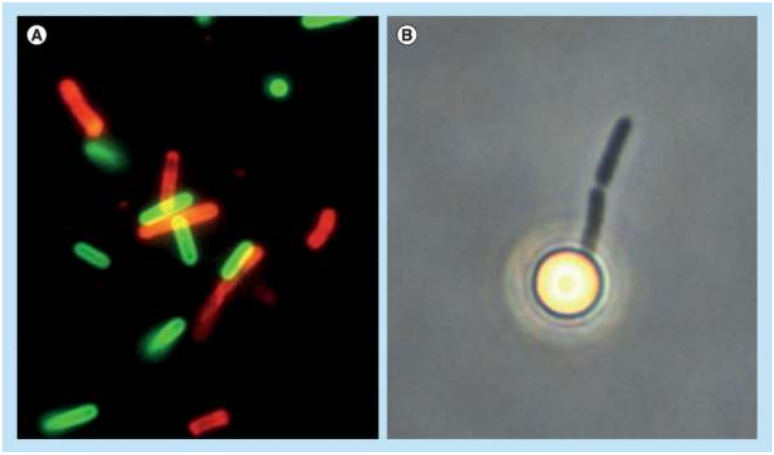

Figure 4. Use of cell wall binding domains for specific differentiation and immobilization of target cells

(A) Differentiation of *Listeria* cells of two different strains by fluorescent microscopy using cell wall binding domains of different serovar specificities fused to either GFP or dsRed as fluorescent markers [Schmelcher M, Unpublished Data]. **(B)** Phase-contrast image of a *Listeria* cell immobilized on the surface of a cell wall binding domain-coated paramagnetic bead [Eichenseher F, Unpublished Data].

Table 1

Molecular engineering and its effects on endolysin properties.

Type of modification	Effect	Examples	Ref.
Exchange of CBD	Switch of regulatory properties	LCA and CLL	[135]
	Swapped cell wall specificity	LC7 and CL7	[67]
		CLC	[136]
		LCL	[137]
		Tsl	[138]
		EAD118_CBDPSA and EADPSA_CBD118	[140]
	Increased lytic activity	LCL	[137]
		EAD118_CBDPSA	[140]
Fusion of two full-length enzymes	Extended lytic spectrum and increased activity	B30-443-Lyso	[102]
Addition of a heterologous EAD to a full-length enzyme	Extended lytic spectrum and increased activity	B30-182-Lyso	[102]
Addition of a heterologous CBD to a truncated enzyme	Extended lytic spectrum and increased activity	λSA2-E-Lyso-SH3b and λSA2-E-LysK-SH3b	[42]
Combination of two heterologous CBDs	Extended binding spectrum	GFP_CBD500-P35 and GFP_CBDP35-500	[140]
Duplication of a CBD	Increased cell wall affinity	GFP_CBD500-500	[140]
	Enhanced lytic activity at high ionic strength	EAD_CBD500-500	[140]
Fusion of an endolysin to a phage minor coat protein	Enhanced solubility	P16-17	[143]
Random mutagenesis	Increased lytic activity and shifted salt optimum	PlyGBS90-1	[79]
Site-directed mutagenesis	Changes in CBD dependence due to altered net charge	PlyBa04-4DFULL and PlyBa04-4DCAT	[21]
	Altered species specificity	L98WCD27L and L98WCD27L1-179	[73]

CBD: Cell wall binding domain; EAD: Enzymatically active domain.