



Review

Bacteriophage endolysins as a response to emerging foodborne pathogens

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Continuous reports on foodborne outbreaks and increasing prevalence of antibiotic-resistant bacteria call for the development of novel preservation techniques that assure the safety of food products. Bacteriophage endolysins are highly active antibacterial peptidoglycan hydrolases that have evolved over millions of years to become the ultimate weapon against bacteria, with potential to be used as a food preservative. We here give an overview of all distinct endolysins encountered so far, we discuss their inherent qualities and review their role in preventing and controlling foodborne pathogens, divulging their potential for future applications.

Introduction

With a continuous increase of bacterial foodborne outbreaks and corresponding costs (FDA, 2009), maintaining

our food quality at an elevated level is of extreme social and economic importance. With 9.4 million foodborne acquired illnesses on a yearly basis in the United States alone, control of foodborne outbreaks has become a vital issue in the last decade (Scallan *et al.*, 2011). In addition, the growing amount of antibiotic-resistant strains is placing patients even more at risk. The increasing consumer awareness has resulted in higher demands for safe food, leading industries to develop more modern production technologies, good manufacturing practices, quality control and hygiene and safety models (such as risk assessment, HACCP and “farm to fork” concepts).

According to several food regulatory entities the common microorganisms that cause foodborne diseases are *Salmonella*, *Escherichia coli* O157:H7, *Campylobacter* and *Listeria*, alongside other, less common infectious species (CDC, 2011; EFSA, 2009). Interestingly, the introduction of chemical preservatives to increase food safety has raised public concern, in part due to the potential risk associated with some of these compounds. Therefore, focus has been directed to more natural antimicrobial alternatives, such as endolysins.

Endolysins (or lysins) are bacteriophage-encoded lytic enzymes that break down the peptidoglycan of the bacterial cell wall during the terminal stage of the bacteriophage reproduction cycle. These enzymes compromise the mechanical strength and resistance of the cell wall that is needed to withstand the internal cytoplasmic turgor (osmotic) pressure, causing bacteriolysis and the subsequent release of the bacteriophage progeny.

A food antimicrobial should have a selective mode of action, should not change the organoleptic characteristics and texture properties of the food product, and should be an innocuous substance for human consumption. Lysins meet all these requirements and therefore seem to be good candidates for the control of foodborne pathogens.

The biotechnological potential of these intriguing enzymes has been discussed previously (Fischetti, 2005b, 2010; Hermoso, Garcia, & Garcia, 2007; Loessner, 2005), however only few have taken on a food-based perspective (Callewaert, Walmagh, Michiels, & Lavigne, 2011).

This review covers key concepts of phage lysin biology, as well as the endolysin–host interactions. It summarizes the endolysin characteristics that are important for efficient food biopreservation and discusses them, in order to allow for a better understanding of their potential as a pathogenic control agent.

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Endolysins

Enzymatic function and substrate recognition on the peptidoglycan network

The rigid peptidoglycan (PGN) layer is responsible for bacterial physical integrity and shape. It is composed of several chains of alternating residues of N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc), connected by β -1,4 glycosidic bonds, linked to a short stem tetrapeptide (Donovan, Dong, et al., 2006). Whereas the carbohydrate backbone is conserved in all bacteria, the peptide moiety made of L- and D-amino acids is only conserved in Gram-negative organisms. In Gram-positives, it is considerably more diverse in terms of length and composition. The amino acid residue in position 3 defines two types of PGN: a *meso*-2,6-diaminopimelic acid type (mDAP-type) in Gram-negative and some Gram-positive species (i.e. *Bacillus* and *Listeria* spp.) and an L-lysine type (Lys-type) typical for Gram-positive organisms. In the mDAP-type the peptide stem is usually directly linked *via* an amide bond, while in the Lys-type they are connected through an interpeptide bridge, such as in pentaglycine (*Staphylococcus aureus*) or dialanine (*Streptococcus pyogenes*) (Fig. 1).

Bacteriophages (or phages) are viruses that specifically infect bacteria. Lacking an independent replication system, they hijack the host's DNA replication and protein synthesis machinery. After replication inside the host, phages are faced with the degradation of the cell wall to release their progeny. To overcome the PGN integrity, most dsDNA phages contain a holin–endolysin based system. Endolysins are enzymes that break down the PGN, however they

generally do not contain signal peptides to direct them toward their substrate. For this to happen, a second hydrophobic membrane-spanning protein – holin – is needed. Holins are responsible for the formation of cytoplasmic membrane patches (Loessner, 2005; Wang, Smith, & Young, 2000) through which endolysins can get access to the murein layer. Endolysins cleave this layer, until the cell loses its capacity to withstand the internal osmotic pressure, ultimately resulting in hypotonic cell lysis. In order to induce cell lysis, endolysins must perform two basic functions: substrate recognition and enzymatic hydrolysis. Endolysins have evolved over millions of years to recognize various ligands within the cell envelope. Yet, due to the well-conserved overall structure of the PGN they are limited in their nature. Depending on their catalytic activity and the bonds that they hydrolyze, endolysins can be divided into different classes (Fig. 1).

Glycosidases cleave the glycan component at the reducing end of GlcNAc (Fig. 1, target 1), as shown for the streptococcal LambdaSa2 lysin (Pritchard, Dong, Kirk, Cartee, & Baker, 2007), or at the reducing end of MurNAc (Fig. 1, target 2), as described for the streptococcal B30 lysin (Pritchard, Dong, Baker, & Engler, 2004). The N-acetyl- β -D-muramidases (often called lysozymes or muramidases) share the same glycan target as the lytic transglycosidases (Fig. 1, target 3), however both deliver different end products: transglycosidases do not work as genuine hydrolases since no water is involved in the cleavage of the glycoside bond, and a 1,6-anhydro bond is formed instead in the muramic acid residue (Vollmer, Joris, Charlier, & Foster, 2008). This is the rarest class

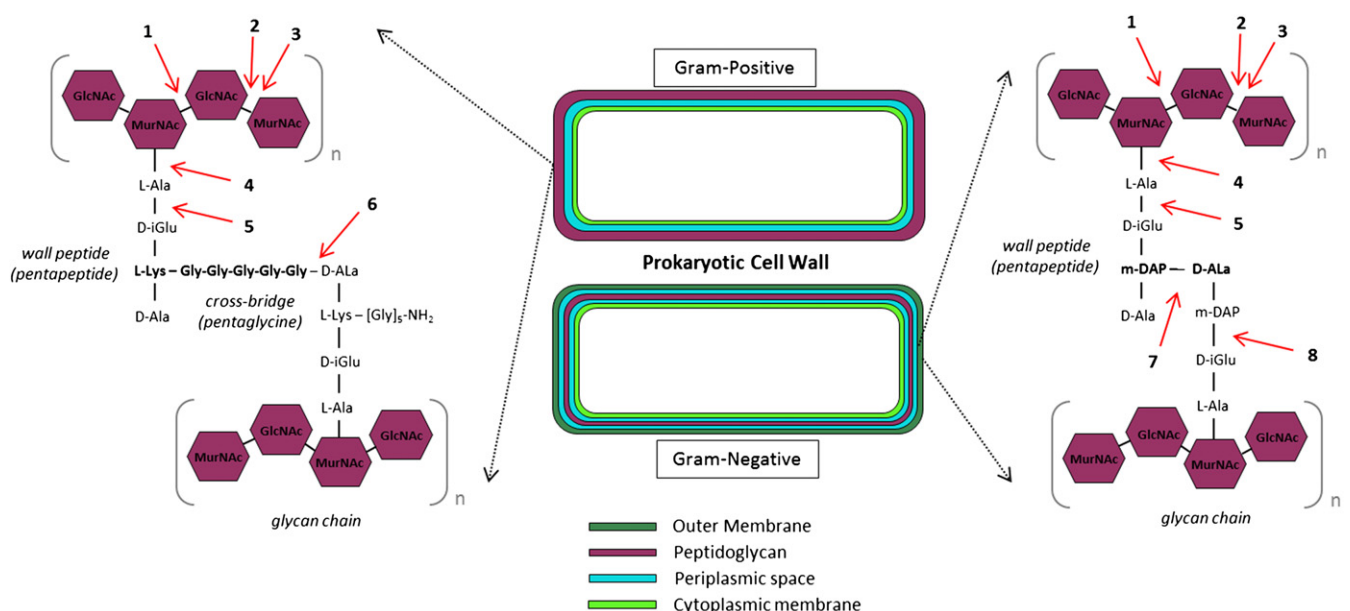


Fig. 1. Schematic representation of the main PGN differences (in bold) between a Gram-positive and Gram-negative bacterium and endolysin mode of action according to their murein activity: 1) N-acetyl- β -D-glucosaminidase; 2) N-acetyl- β -D-muramidase; 3) lytic transglycosylase; 4) N-acetylmuramoyl-L-alanine amidase; 5) L-alanyl-D-glutamate endopeptidase; 6) D-alanyl-glycyl endopeptidase; 7) D-alanine-D-meso-DAP endopeptidase and 8) D-glutamyl-m-DAP endopeptidase (no endolysin has been reported for class 7 and 8).

among the phage lysins and is found in the *Pseudomonas aeruginosa* phage phiKZ gp144 lysin (Fokine, Miroshnikov, Shneider, Mesyanzhinov, & Rossmann, 2008). Distinct classes of amidases catalyze the hydrolysis of the critical amide bond between MurNAc and the L-alanine (Fig. 1, target 4), separating the glycan strand from the stem peptide. Therefore, amidases are predicted to cause the strongest destabilization effect in the PGN. In addition, distinct classes of endopeptidases and carboxypeptidases attack the LD- and DD-bonds in the stem peptides, as has been demonstrated for *Listeria* Ply500 and the Ply118 L-alanyl-D-glutamate endopeptidases (Loessner, Wendlinger, & Scherer, 1995) (Fig. 1, target 5). Other endopeptidases, like the staphylococcal phi11 D-alanyl-glycyl endopeptidase, also cleave within peptides that crosslink the cell wall (Navarre, Ton-That, Faull, & Schneewind, 1999) (Fig. 2, target 6). The muramidase and amidase groups target the most conserved bonds in the PGN and seem to be the most widely spread (Fischetti, 2008).

As a result of their different enzymatic functions, the lytic spectrum of Gram-positive phage endolysins is considered to be limited to the bacterial species from which the phage is derived. Examples of such restricted activity have been reported for the pneumococcal bacteriophage

lytic enzyme Pa (Loeffler, Nelson, & Fischetti, 2001) and the *Bacillus anthracis* phage endolysin PlyG (Schuch, Nelson, & Fischetti, 2002). Furthermore, the *Staphylococcus* bacteriophage endolysin LysH5 only leads to lysis of *S. aureus* and *Staphylococcus epidermidis* (Obeso, Martinez, Rodriguez, & Garcia, 2008) and is not active against several lactic acid bacteria and strains belonging to *Bacillus*, *Streptococcus*, *Clostridium*, *Listeria*, and *Enterococcus* spp., as expected due to their low degree of PGN homology. The pneumococcal phage endolysin Cpl-1 recognizes choline decorations on teichoic acids, resulting in a very narrow activity spectrum (Perez-Dorado et al., 2007). Nevertheless, endolysins with a broader lytic spectrum can also be found. An example is PlyV12, produced by the Enterococcal bacteriophage phi1. This endolysin displays activity on 14 *Enterococcus faecalis* and *Enterococcus faecium* strains, on pathogenic streptococci, including *S. pyogenes* (Group A, B and C) and on *S. aureus* strains (Yoong, Schuch, Nelson, & Fischetti, 2004). Endolysin Lyb5, from the *Lactobacillus fermentum* temperate bacteriophage phiPYB5, exhibits activity on both Gram-positive (*S. aureus*, *Bacillus subtilis* and several lactic acid bacteria) and Gram-negative bacteria (e.g. *E. coli* and *Salmonella typhimurium*) (Wang, Kong, & Zhang,

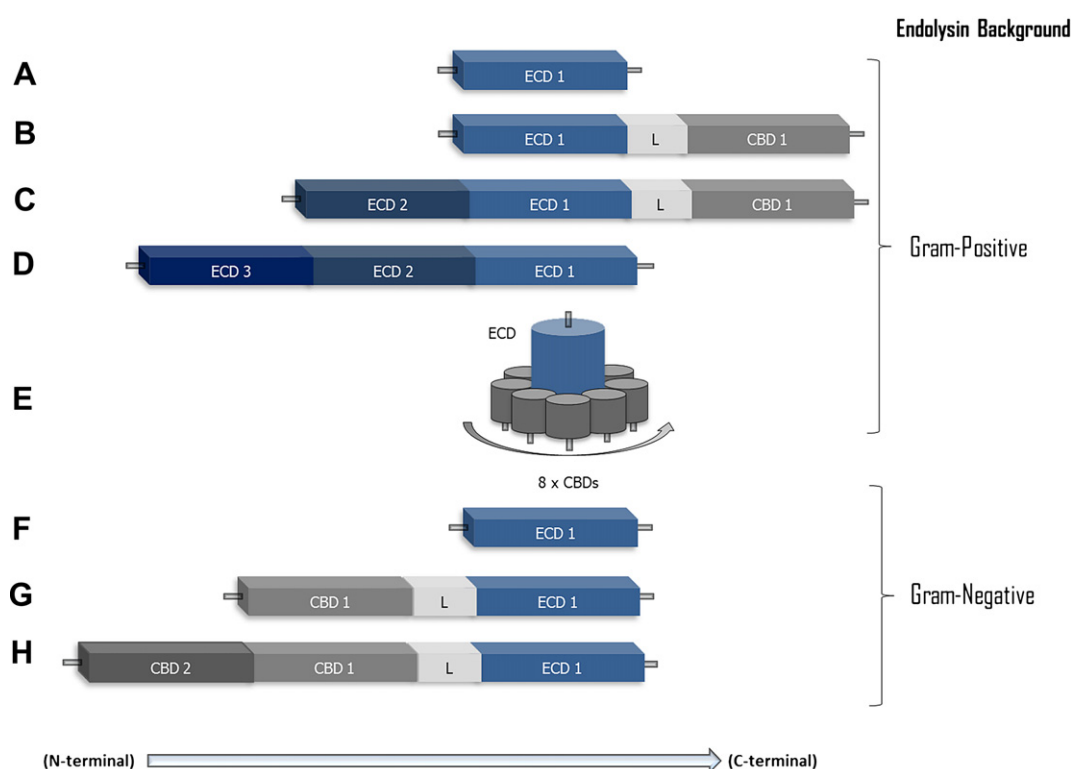


Fig. 2. Overview of different reported molecular structures for endolysins. A. ECD only (globular enzyme, atypical in Gram-positive endolysins); B. Bimodular structure with N-terminal ECD and a C-terminal CBD; C. Trimodular structure with two N-terminal ECDs and a C-terminal CBD; D. Trimodular structure with three ECDs; E. Multimeric structure composed of a single heavy chain and eight light chains; F. ECD only (globular enzyme, typical in Gram-negative endolysins) G. Bimodular structure with N-terminal CBD and a C-terminal ECD; H. Trimodular structure with two N-terminal CBDs and a C-terminal ECD. Abbreviations: ECD, enzyme catalytic domain; CBD, cell wall binding domain. Adapted from Briers, Volckaert, et al. (2009).

2008). The *Lactobacillus helveticus* bacteriophage phi-0303 endolysin Mur-LH is active on several lactic acid bacteria, but also on more distant species like *B. subtilis*, *E. faecium* and *Pediococcus acidilactici* (Deutsch, Guezenc, Piot, Foster, & Lortal, 2004). Amidase classes of endolysins may represent a broader lytic spectrum compared to other classes, since the amide bond between the N-acetylmuramic acid residue and L-alanine is conserved among several bacterial species (Navarre et al., 1999).

Finally, we note that thus far only a few endolysins have been identified that are able to degrade pseudomurein, the peptidoglycan component of methanogenic archaea. To date, no enzymes have been found that can cleave the β -1,3 glycosidic bond of pseudomurein, and only two endoisopeptidases, PeiW from the methanogenic prophage *Methanothermobacter wolfeii* psiM100 and PeiP from *Methanothermobacter marburgensis* psiM2, have been identified that cleave between the ϵ -amino group of L-lysine and the carboxyl group of an L-alanine residue within pseudomurein (Visweswaran, Dijkstra, & Kok, 2010).

Natural molecular structures inherited

Depending on their origin, endolysins can vary in their molecular structure, either modular or globular, and in their domain orientations. An illustration of all molecular arrangements encountered so far is represented in Fig. 2.

Gram-positive endolysins possess a typical, well-defined modular architecture, with an enzymatic catalytic domain (ECD) at the N-terminal and a cell wall binding domain (CBD) at the C-terminal end, separated by a short linker (Fischetti, 2008; Loessner, 2005). While the ECD is responsible for the cleavage of the bonds in the PGN of the bacterial cell wall, the CBD confers specificity in substrate recognition and subsequent binding to the cell wall. Some endolysins have even acquired more than one independent ECD, such as the endopeptidase/muramidase from the *Streptococcus agalactiae* bacteriophage B30 (Pritchard et al., 2004), the muramidase/endopeptidase of a pneumococcal phage Cpl-1 lytic enzyme (Hermoso et al., 2003), the endopeptidase/amidase of the phage phi11 lysin (Navarre et al., 1999) and the endopeptidase/glucoamidase of a streptococcal LambdaSa2 lysin (Donovan & Foster-Frey, 2008). However, the presence of two catalytic domains does not necessarily mean that they are equally active: the D-glutaminyll-L-lysine endopeptidase of the streptococcal lambdaSa2 was found to be responsible for almost the entire hydrolytic activity of the protein (Donovan & Foster-Frey, 2008).

The presence of three ECDs has only been described for the staphylococcal phage187 lysin (Ply187), which harbors an endopeptidase, an amidase and a glucosaminidase module. However, not all lytic activities were experimentally demonstrated (Cheng, Nelson, Zhu, & Fischetti, 2005). The acquisition of such an organization has probably evolved in order to adapt to changes in environmental conditions. Besides endolysins with a modular organization,

globular proteins with a single ECD also occur (e.g. T4 lysozyme (Matthews & Remington, 1974)). Another interesting example is the streptococcal C1 bacteriophage lysin. While in general endolysins have a molecular mass of 25–40 kDa (Fischetti, 2008), this unique lysin weighs 114 kDa. It is a multimeric PlyC that consists of one heavy chain representing the catalytic site (PlyCA), and eight PlyCB chains that bind the cell wall (Nelson, Schuch, Chahales, Zhu, & Fischetti, 2006). Comparison of the amino acid sequences of Gram-positive lytic enzymes of the same enzyme class reveals high homology within the N-terminal catalytic region and very little homology within the C-terminal cell binding region.

In contrast to their Gram-positive counterparts, endolysins active against Gram-negatives generally display a globular structure (i.e. only contain the ECD) and rarely show a modular organization. With no exceptions, the few endolysins with a modular structure all have inverted molecular orientations compared to Gram-positive endolysins, with the ECD at the C-terminal and the CBD at the N-terminal side (e.g. KZ144 of phiKZ (Briers et al., 2007)). An even more unusual endolysin is OBPgp279, belonging to the *Pseudomonas putida* phage OBP, which is predicted to have two CBDs (Cornelissen et al., 2011). While it is shown that CBDs of endolysins targeting Gram-positives enhance the substrate affinity of the enzyme, those found in Gram-negatives, like endolysin KZ144 and EL188, show a broad binding spectrum (Briers et al., 2007).

The fact that CBDs only occur in endolysins of Gram-positive origin and not in their Gram-negative counterparts may have an evolutionary explanation. The PGN in Gram-positive bacteria is exposed to the surface. If enzymes deprived of a CBD spill out after cell lysis, they would have a good chance of killing surrounding bacteria before the progeny phage particles could start a new infection cycle (Fischetti, 2008). Subsequently, it is believed that these enzymes have evolved to bind irreversibly to their cell wall ligand by means of a CBD, so that they are not released after cleavage of the target site (Loessner, Kramer, Ebel, & Scherer, 2002). Converging with this is the theoretical catalytic capacity of a single enzyme to cleave enough bonds to kill an organism and the extremely high, nanomolar range, affinity of their CBDs (Loessner et al., 2002). This suggests that they are single-use enzymes only. Such phenomenon would drive phages to produce endolysins in just the right amount to lyse the cell. In contrast to Gram-positive bacteria, Gram-negative cells are resistant to external lysin treatment (“lysis from without”), due to their protective outer membrane. As such, there is no evolutionary pressure to acquire CBDs, making the observation of the few modular endolysins from Gram-negative infecting phages extremely interesting. More specifically, the few modular endolysins active against Gram-negatives were isolated from the *P. aeruginosa* phages phiKZ and EL, *P. putida* phage OBP (with genome sizes of 280 kb, 211 kb, and 207 kb, respectively) (Cornelissen et al.,

2011), *Pseudomonas chlororaphis* 201phi2-1 (316 kb) (Thomas *et al.*, 2008), *Salmonella enteritidis* phage PVPSE1 (145 kb) (Santos *et al.*, 2011) and *Campylobacter coli* phage phiCcoIBB35 (204 kb, not published), all belonging to the Myoviridae family. These data suggest that this rare modular organization might be restricted to Myoviridae giant phages.

Endolysin architectural structure engineered

The compelling need to develop even more efficient endolysins, and the relative independence of the modular domains within endolysins, has led to the creation of new hybrid enzymes. In this approach, the emphasis is laid on adding, truncating or swapping endolysin domains to obtain enzymes with optimized applications. A handful of studies have used this strategy with the aim to increase the specificity or efficiency of endolysin activity. Pioneer work focused on exchanging catalytic domains of a *Streptococcus pneumoniae* phage lysin, which resulted in endolysins with the same binding characteristics but with multiple PGN targets (Garcia, Garcia, Garcia, Sanchez-Puelles, & Lopez, 1990). Since then, other studies have followed by using the same proof of principle, as for instance with the pneumococcal phage CPL1 lysozyme (Diaz, Lopez, & Garcia, 1990). The Staphylococcal P16 endolysin was modified in a similar way to overcome insolubility problems (Manoharadas, Witte, & Blasi, 2009). For Gram-positive endolysins, structural engineering has resulted in mixed successes. For instance, CBD truncation of the endolysins Ply118 and Ply500 (Loessner *et al.*, 2002) or the *Clostridium perfringens* bacteriophage phi3626 lysin (Zimmer, Vukov, Scherer, & Loessner, 2002) abolished antibacterial activity, while for the Mur endolysin lytic activity remained unaltered (Vasala, Valkkila, Caldentey, & Alatossava, 1995). In other studies higher bacteriolytic activity was achieved (Gaeng, Scherer, Neve, & Loessner, 2000; Loessner, Gaeng, & Scherer, 1999; Loessner, Gaeng, Wendlinger, Maier, & Scherer, 1998; Low, Yang, Perego, Osterman, & Liddington, 2005) and in the case of the CBD-truncated LysK and PlyGBS lysins a 2-fold and approximately 25-fold increase in muralytic activity was attained, respectively (Cheng & Fischetti, 2007; Horgan *et al.*, 2009). Innovative work on the detection of biological weapons has been carried out, using the high affinity and sensitivity of a CBD of a gamma-phage lysin (*B. anthracis*, the causative agent of anthrax) (Fujinami, Hirai, Sakai, Yoshino, & Yasuda, 2007). Similarly, CBDs were applied to bind and capture pathogens present in food samples (Fujinami *et al.*, 2007; Kretzer *et al.*, 2007). Interestingly, complete CBD sequences are not always needed for this purpose, as a 10 amino-acid motif of the phage lysin PlyG CBD was shown to be sufficient for the specific detection of *B. anthracis*, when coupled to fluorescent quantum dots (Sainathrao, Mohan, & Atreya, 2009).

At least two modular endolysins were demonstrated to be active against Gram-negative bacteria in the absence

of their N-terminal CBDs (Walmagh, Briers, Santos, Azeredo, & Lavigne, 2012). Interestingly, despite that CBDs are responsible for targeting the bacterial cell wall, they do not always seem to be essential for endolysin antibacterial activity. This finding has been observed for both Gram-positive and Gram-negative bacteria, but awaits a molecular explanation. The currently described modular endolysins, active against Gram-negative bacteria, have all presented a wide lytic spectrum range (Briers *et al.*, 2007). This suggests a lack of bacterial specificity in contrast to their Gram-positive counterparts. However, their CBDs can still be applied as permeabilizing biomarkers, when fused to marker proteins such as fluorescent proteins, to access the Gram-negative outer membrane (Briers, Schmelcher, *et al.*, 2009; Briers, Volckaert, & Lavigne, 2009).

Phage lysins in food biotechnology

Endolysin features for food safety applications

Foodborne diseases are an increasing matter of concern, especially in view of the alarming emergence of antibiotic-resistant bacterial strains. Therefore, attention has been drawn to bacteriophage therapy as alternative bacteriolytic agents to synthetic antimicrobials. Recently, two commercial bacteriophage cocktails that battle Listeriosis, Listex™ P100 (Microcos) and ListShield (Intralytix), have received FDA approval (Shuren, 2006). However, in Europe it remains uncertain whether phages can be considered as processing aids or as decontaminants/additives (Teufer & Von Jagow, 2007). In addition, the need to select virulent phage to avoid transduction, the threshold requirements of the host and the potential development of bacterial strains resistant to phages are the main obstacles when considering phage as an antimicrobial for the food industry. Phage endolysins lack such disadvantages and therefore represent a promising alternative for controlling foodborne pathogens.

Before considering the application of phage lysins as a means to control foodborne diseases, an analysis of their stability on food products and food processing facilities should be considered, alongside other aspects concerning consumer safety. Biocontrol agents need to withstand all aspects encountered in the foodborne matrix, such as stability under the physicochemical conditions in which they are applied. They should be commercially attractive to consider investment, and finally, they should be allowed as a biocontrol product by the international food regulatory agencies and accepted by the consumer.

Host specificity

Endolysins only cleave PGN linkages that are exclusively present in bacteria; however by displaying dissimilar lytic spectra they can be exploited differently. When a wide range of bacteria has to be controlled, endolysins with a broad host range will be required. For instance, in agriculture, endolysins targeting different bacterial taxa could play an important role as biopesticides, preventing tomato scabs,

wilts and spots caused by *Streptomyces scabies*, *Clavibacter michiganensis* and *Xanthomonas campestris*, respectively. In milk processing plants, endolysins could act as food sanitizers targeting thermophilic bacteria (e.g. *Bacillus* spp. and *Paenibacillus* spp.) and psychrotolerant *Pseudomonas* spp., preventing spoilage of pasteurized milk and extending the shelf-life of food products.

Endolysins with a narrower range of action, targeting a specific or at least closely related bacterial species or genus, could prove useful for the elimination or control of specific pathogens or spoilage organisms. In livestock, endolysin – prebiotic – products could be administered to control enteric diseases in the cattle and poultry gastro-intestinal tract (e.g. with *C. perfringens* Ply2626 lysin on poultry), while leaving the gut microflora unaltered (Zimmer et al., 2002). Alternatively, endolysins can control mastitis-causing bacteria in mammary glands of dairy cattle (e.g. with *Streptococcus uberis* Ply700 and *S. aureus* LysH5 endolysins) (Celia, Nelson, & Kerr, 2008; Obeso et al., 2008), preventing cross-contamination of food during milking and slaughtering procedures. In industrial fermentations, endolysins like Ply511 could be secreted *in situ* in modified starter cultures, to control *Listeria monocytogenes* spoilage in milk (Gaeng et al., 2000), or added as a food additive, like the clostridial-specific CTP1 lysin preventing cheese spoilage and blowing (Mayer, Payne, Gasson, & Narbad, 2010).

Temperature

The effect of thermal stress on endolysins can be variable. It is important to determine their antimicrobial activity under the different temperatures that can be found in food systems (e.g. chilled food of approximately 4 °C) to allow for efficient biocontrol. Surprisingly, endolysins have been shown to perform at a wide temperature range. While the bacteriophage T4 lysozyme retains only a minor fraction of its activity after a 5 min treatment at 65 °C (Nakagawa, Arisaka, & Ishii, 1985), KZ144 and EL188 endolysin activity remains high even when exposed for 10 min to 50 °C (Briers et al., 2007). Others are shown to be stable for 30 min at 45 °C (Loeffler, Djurkovic, & Fischetti, 2003), or exhibit only a 30% decrease in activity when exposed for 30 min to 90 °C (Schmelcher, Waldherr, & Loessner, 2011). Even higher thermostability has been described for the lysozyme domain of gp36 from a *P. aeruginosa* phage phiKMV, which is resistant to temperatures up to 100 °C (Lavigne, Briers, Hertveldt, Robben, & Volckaert, 2004). However, it is likely that phages isolated from more thermostable bacteria will contain endolysins that are even more thermostable. Good examples of sources of potentially thermostable endolysins are *Bacillus* phage W1 and *Geobacillus* phage E1, isolated from deep-sea thermophilic bacteria, or the bacteriophage phiTMA isolated from the extreme thermophile *Thermus thermophilus*, originating from hot springs (Liu, Wu, Song, Zhang, & Xie, 2006; Tamakoshi et al., 2011). Endorsing this hypothesis

is the characterized *Thermus aquaticus* phage phiIN93 endolysin with an optimal activity between 60 and 120 °C (Matsushita & Yanase, 2008). Some bacteria, such as *Listeria* spp., are able to multiply even in refrigerated conditions. However, little is known about endolysin activity on this side of the temperature spectrum. Activity at temperatures as low as 2 °C has been described for the bacteriophage phiLM4 lysin (Gasson, 1992). Recently, an endolysin LysZ5 report showed the ability to reduce *Listeria* contamination at 4 °C on soya milk (Zhang, Bao, Billington, Hudson, & Wang, 2012). It is therefore estimated that with further phage characterization, novel thermo- and cryo-resistant endolysins will be identified and new potential food applications that require thermal processing will be established.

pH tolerance

Phage lysin studies have indicated that their optimum pH usually lies within the range of 4.0–6.0 (Borysowski, Weber-Dabrowska, & Gorski, 2006). However, some lysins retain significant antibacterial activity at pH 7.4 (blood pH) (Fischetti, 2004; Jado et al., 2003; Loeffler et al., 2003). In addition, the endolysin PlyPH, with a specific activity against *Bacillus cereus* and *B. anthracis* strains, remains active over an unusually broad pH range of 4–10.5 (Yoong, Schuch, Nelson, & Fischetti, 2006).

Processing environments

Potential problems in food processing units associated with bacterial biofilms – the formation of an aggregate of microorganisms as a result of bacterial surface adherence – can be overcome by the use of endolysins. Despite the higher resistance of biofilms to antimicrobials, compared to planktonic cells, two staphylococcal phage lysins were shown to successfully hydrolyze staphylococcal biofilms (Sass & Bierbaum, 2007; Son et al., 2010).

On food environments, studies have demonstrated bacterial elimination from food samples such as milk (with a high content of lipids and proteins, and a pH ranging from 6.4 to 6.8). In addition, endolysin activity has been shown in the presence of nonionic detergents (i.e. hard water and organic compounds). Furthermore, its potential as an enzymatic disinfectant on surfaces has been investigated (Hoopes et al., 2009), as well as its antimicrobial properties (Donovan, Lardeo, & Foster-Frey, 2006; Garcia, Martinez, Rodriguez, & Rodriguez, 2010). Still, enzyme stability should be measured taking into account all possible enzyme antagonists and catalytic toxic effects that can be encountered in all food matrices (e.g. proteolytic enzymes).

The use of non-thermal treatment approaches can be synergistically used with bactericidal compounds to contribute to food stabilization and shelf-life prolongation. This was demonstrated when high hydrostatic pressure (HHP) was combined with lysozymes in the inactivation of the Gram-positive bacteria *Listeria innocua* and *S. aureus* (Masschalck, Deckers, & Michiels, 2002). HHP

can also be used to facilitate outer membrane permeabilization by endolysins on Gram-negative bacteria. This approach was proven to efficiently inactivate several Gram-negative pathogens, without loss of enzyme activity even when exposed to harsh treatment at 600 MPa and 60 °C for 15 min (Briers *et al.*, 2008; Masschalck, Van Houdt, Haver, & Michiels, 2001; Nakimbugwe, Masschalck, Atanassova, Zewdie-Bosuner, & Michiels, 2006). HHP can be applied as an alternative to heat pasteurization, the more conventional food preservation technique. It even allows a better retention of product properties because it can be applied at low temperatures. With the current state of this technology, its use could be limited only by financial inviability, as its implementation requires high investment costs.

Antimicrobial efficiency

The potent antimicrobial power of endolysins is notable. Nanogram quantities were able to eliminate bacteria from suspensions in seconds (Loeffler *et al.*, 2001; Nelson, Loomis, & Fischetti, 2001). In addition, affinity detection methods using nanomolar amounts of CBDs have been described (Loessner *et al.*, 2002). To date, no other known biological compound has been found to kill microorganisms this quickly. Even the lysis of dead cells has been reported (Sonstein, Hammel, & Bondi, 1971), although this is considered highly unfavorable as it diverges the enzyme away from the real target: living bacteria. A positive aspect is the possibility to eradicate antibiotic-resistant bacteria, as was shown on methicillin-resistant and multidrug-resistant *S. aureus* (MRSA) strains (endolysin LysK and endolysin MV-L, respectively) (O'Flaherty, Coffey, Meaney, Fitzgerald, & Ross, 2005; Rashel *et al.*, 2007), or penicillin-resistant *S. pneumoniae* (Loeffler *et al.*, 2001). It was also shown to work well against *E. faecalis* and *E. faecium* strains resistant to vancomycin (endolysin PlyV12) (Yoong *et al.*, 2004). To further improve the antimicrobial efficacy, synergistic methods using an endolysin cocktail or a combination with antibiotics can be employed to eliminate bacteria less accessible to antibiotics. As a result, the emergence of lysin/antibiotic resistant mutants would slow down. Consistent with this, antibiotic-resistant *S. pneumoniae* strains have been targeted using Cpl-1 and Pal lysins having the same target specificity, but different catalytic activities *in vitro* (Loeffler & Fischetti, 2003) and *in vivo* (Jado *et al.*, 2003). Synergistic effects between lysins and antibiotics have also been demonstrated with the Cpl-1 bacteriophage lytic enzyme and penicillin or gentamicin against *S. pneumoniae* strains with different levels of susceptibilities to penicillin (Djurkovic, Loeffler, & Fischetti, 2005). The staphylococcal lysin MV-L was also used in combination with the glycopeptide antibiotics vancomycin or teicoplanin against the vancomycin-intermediate *S. aureus* (VISA) (Rashel *et al.*, 2007).

Long-term storage (shelf life)

A good food antimicrobial extends the shelf-life of food products. Several endolysins demonstrate an extraordinarily long-term stability, such as Cpl-1, which showed no loss in activity over 6 months at 4 °C and 3 weeks at 37 °C (Loeffler *et al.*, 2003). Other examples of endolysins with a long-term stability are KZ144 and EL188, which can be stored for 4 months at 4 °C while retaining full activity (Briers *et al.*, 2007) (see also sections *Temperature* and *pH tolerance* for more information on factors determining the endolysin's shelf-life properties).

Costs

The production costs of endolysins are expected to be high with the current technology, which may constitute the most significant barrier to their application as an alternative to phages or antibiotics. However, in the food industry, a cost-efficient production of enzymes should not be considered an insurmountable obstacle, since a wide variety of enzymes are commercially available and produced in kg to ton quantities for application as enzyme supplements (baking, brewing, cheese flavoring, etc.). Technological developments for more efficient expression systems would make the option to use endolysins as food control agents a financially appealing one. Also, *in situ* expression of natural antimicrobials such as endolysins in transgenic grains, plant leaves, crops and even animals may eventually reduce costs. For lysozymes all these scenarios have been successfully applied to prevent pathogenic contamination. For phage endolysins however only expression in transgenic potatoes and in starter cultures has been reported (During, Porsch, Fladung, & Lorz, 1993; Gaeng *et al.*, 2000). Therefore, much work still remains to be done.

Avoidance and resistance

Repetitive exposure of bacteria grown on agar plates to low concentrations of lysin did not lead to the recovery of resistant strains; neither did bacterial resistance occur after several cycles of exposure to low concentrations of enzyme in liquid conditions (Loeffler *et al.*, 2001). In contrast, several antiviral mechanisms of bacteria against phages have been described (Labrie, Samson, & Moineau, 2010). It has been postulated that the lack of bacterial resistance toward endolysins is due to their unique mode of action. To avoid being trapped inside the host, phages have evolved to produce enzymes such as endolysins, targeting essential molecules that cannot be altered by bacteria. As a result, bacterial resistance is rare (Fischetti, 2004, 2005a; Loeffler *et al.*, 2001). This is the case for the endolysins of *Pneumococcus* phages that bind to choline, an amino alcohol present in PGN and indispensable for pneumococcal viability (Fernandez-Tornero *et al.*, 2005). Another example is the action of an endolysin of the *Streptococcus* group A phage that binds to polyribose, which is a molecule indispensable for the growth of these bacteria (Fischetti, 2003). To minimize the occurrence of resistance, a selection

of multiple endolysins targeting different substrates could be used, as was shown by the synergistic effect of two phage lytic enzymes on penicillin-resistant *S. pneumoniae* strains (Loeffler & Fischetti, 2003).

Consumer's acceptance

The use of genetically modified organisms (GMOs) as cell factories for endolysin production may raise public concern, in case application in consumer products is envisaged. A survey in 2010 showed that the amount of consumers that are very or extremely concerned about eating GMO food marginally decreased (3%) compared to two years before (Deloitte, 2010). GMOs have entered our lives the last few decades, however European and American governing authorities have not yet managed to generalize legislation regarding their use (Varzakas, Arvanitoyannis, & Baltas, 2007). Regarding endolysins, this discussion applies more to the use of GMOs as a whole and to what extent GMOs and their "foreign" DNA can be spilled into the environment. Concerning the use of their translated end product, endolysins can be considered as processing aids or as food additives, in which case stringent tests to prove the absence of toxins, allergens, or other hazardous compounds will determine their safety (Law, 2002). Several successful preclinical treatments with endolysins have been carried out in animal models and thus far no potential toxicity was observed (Loeffler et al., 2003; McCullers, Karlstrom, Iverson, Loeffler, & Fischetti, 2007).

Table 1 presents an overview of the main factors when considering employing an endolysin food-based methodology.

Application of phage lysins as biocontrol agents

Based on phage lysis specificity and host recognition, several food applications have been stipulated to treat or prevent spoilage. Several reports on the antimicrobial application of endolysins along the food processing line have been conducted, however much work remains to be done (Table 2). On the primary commodities point of application, secretion of the foreign T4 endolysin into the intercellular spaces of transgenic potato plants effectively demonstrated plant resistance to the phytopathogenic bacterium *Erwinia carotovora* (During et al., 1993). Expression of the bacteriophage FEa1h endolysin resulted in growth inhibition of *Erwinia amylovora* on pears (Kim, Salm, & Geider, 2004). *S. aureus* biocontrol in milk has been proposed by expressing endolysins in transgenic cows (Donovan, Lardeo, et al., 2006). Regarding *S. aureus* biofilm removal, a proof of concept was given using recombinant bacteriophage phi11 lysin (Sass & Bierbaum, 2007). As a prophylactic measure, PlyC endolysin significantly reduced *Streptococcus equi* (Hoopes et al., 2009). Ply3626 has been proposed as an agent to control the *C. perfringens* population by direct addition to food, raw products and feed, to fight the development of this pathogen in intestines of poultry (Zimmer et al., 2002). In a study, aiming to

Table 1. Factors affecting application in food-based methodologies.

	Effect
▶ Process-related	
• Low probability to develop resistance	+
• Both specific and broad activity spectrum	+
• Active under refrigerated conditions (for chilled products)	+
• Efficacy for biofilm removal on food process surfaces (e.g. on Gram-positive cells)	+
• Thermostability for thermal processing products	+
▶ Food-related	
• Susceptible for inactivation on food matrix extreme pHs	–
• Sensitive to host-proteolytic activity	–
• Organoleptic characteristics remain unchanged	+
• Extend antimicrobial shelf-life	+
• Not self-replicating antimicrobial agent	–
• Efficient on antibiotic resistant pathogens	+
• Limited applications on Gram-negative pathogens	–
▶ Food regulatory acceptance	
• Apparent safe status (non-toxic)	+
• Not self-replicating antimicrobial agent (better acceptance)	+
• Regulatory and consumer acceptance	+/-
▶ Economic/marketing impact	
• Efficient antimicrobial (at nanoscale level)	+
• New mode of action (cleavage of PGN)	+
• Susceptible to protein engineering to achieve better performance	+
• Relatively expensive to produce	–

selectively protect dairy products, Gaeng et al. examined the possibility to recombinantly introduce an endolysin gene in *Lactococcus lactis*. Their work resulted in the production of dairy starter cultures with biopreservation properties against *L. monocytogenes* contaminations (Gaeng et al., 2000). Regarding mastitis control programs, bacteriophage lysin Ply700 was shown to kill *S. uberis* in milk, but more tests are needed to determine its efficiency on controlling *S. uberis* mastitis (Celia et al., 2008). Finally, Zhang and coworkers found that the endolysin LysZ5 from the *L. monocytogenes* phage FWLLm3 was able to reduce Listerial contamination in soya milk at refrigeration temperatures (4 °C) (Zhang et al., 2012).

In food processing environments, more particularly in dairy manufacturing, work has been developed in an attempt to kill *S. aureus* in pasteurized milk using staphylococcal phage endolysin LysH5 (Obeso et al., 2008). In fermented products, endolysins can be used to avoid contamination in starter cultures. Mayer and colleagues showed the potential of the CTP1 endolysin to control late blowing during cheese fermentation without affecting the fermentation microbiota (Mayer et al., 2010). On a different approach, a *L. lactis* endolysin expression system was created to accelerate cheese ripening (de Ruyter, Kuipers, Meijer, & de Vos, 1997).

Table 2. Summary of *in situ* or possible endolysins food-based applications.

Targeted pathogen	Endolysin	Sample matrix (aim)	Outcome/reducing effect	Refs.
<i>E. carotovora</i>	T4 lysozyme	Transgenic potatoes	Endolysin secretion in transgenic potato plants effects a resistance against <i>E. carotovora</i> at low expression levels	(During et al., 1993)
<i>E. amylovora</i>	phiEalh lysin	Pears surface	Ooze formation and necrosis were retarded or inhibited after inoculation with <i>E. amylovora</i>	(Kim et al., 2004)
<i>S. aureus</i>	phi11 lysin	Bovine milk (aim)	Up to 2 log reduction on coagulase-negative staphylococcus	(Donovan, Lardeo, et al., 2006)
<i>S. equi</i>	PlyC lysin	Horse-related material surfaces	Aerosolized PlyC could sterilized 7 of 8 common stable equipment	(Hoopes et al., 2009)
<i>C. perfringens</i>	Phi3626 lysin	Poultry intestines (aim)	Active to all 48 <i>C. perfringens</i> strains tested, not affecting other clostridia and bacteria belonging to other genera	(Zimmer et al., 2002)
<i>L. monocytogenes</i>	Ply511 lysin	Milk fermentation (aim)	Possibility to secrete an active Listeria endolysin although their antagonistic activity has not been assessed	(Gaeng et al., 2000)
<i>S. uberis</i>	Ply700 lysin	Bovine milk	Ply700 (50 µg/ml) killed 81% of <i>S. uberis</i> in milk	(Celia et al., 2008)
<i>S. aureus</i>	LysH5 lysin	Pasteurized milk	The addition of 160 U/ml (88 µg/ml) of LysH5 to pasteurized milk reduced the viable counts to undetectable levels in 4 h	(Obeso et al., 2008)
<i>C. tyrobutyricum</i> <i>L. lactis</i>	phiCTP1 lysin phiUS3 lysin/holin	Semi-skim milk Cheese milk	One log reduction unit Fourfold increase in release of L-Lactate dehydrogenase activity contributing for accelerate cheese ripening	(Mayer et al., 2010) (de Ruyter et al., 1997)
<i>L. monocytogenes</i>	CBD118 and CBD500	Meat, poultry, fish, dairy and ready-to-eat products	Listeria separation from food samples using CBDs revealed to more sensitive and less time consuming (only 48 h versus 96 h) from standardized methods	(Kretzer et al., 2007)
<i>L. monocytogenes</i>	CBDP40	Milk and cheese	Cells from different <i>Listeria</i> strains were removed and identified from food samples	(Schmelcher et al., 2010)
<i>L. monocytogenes</i>	CBDP40	Milk and cheese	Cells from different <i>Listeria</i> strains were removed and identified from food samples	(Schmelcher et al., 2010)
<i>L. monocytogenes</i>	Lys	Soya milk	More than 4 logs reduction after 3 h incubation at refrigerated temperature (4 °C)	(Zhang et al., 2012)

By virtue of their high specificity toward the targeted bacteria, endolysins can be further used as identification and capture systems (Fujinami et al., 2007). In this context, high immobilization and removal of *L. monocytogenes* from artificially and naturally contaminated samples was achieved using a CBD-based magnetic separation method (Kretzer et al., 2007). Relying on their high efficiency, even different *Listeria* strains could be recovered from milk and cheese using a CBD cocktail with different fluorescent markers (Schmelcher et al., 2010).

Resistant Gram-negative foodborne bacteria

So far, the main target for endolysins in food systems has been Gram-positive microorganisms, mostly because of their enhanced susceptibility to endolysins due to the lack of a protective outer membrane. Since foodborne contaminations are mostly caused by Gram-negative bacteria,

and in view of their conserved PGN structure, the application of lysins on these bacteria is considered to be the “holy grail” in endolysin control of foodborne bacteria. Possible strategies that can be foreseen to sensitize Gram-negative bacteria are outer membrane permeabilization using physical processing, the use of outer membrane permeabilizing compounds or the covalent modification of endolysins.

Although permeabilization agents have been extensively reviewed by Vaara (Vaara, 1992), so far only one successful study using an EDTA treatment coupled with the endolysin EL188 to control *P. aeruginosa* cells has been reported. The addition of short hydrophobic amino acids motifs, predicted to target the outer membrane bilayer, constitutes a promising strategy to lyse Gram-negative bacteria. It was found that both *Bacillus amyloliquefaciens* phage lysin and the *Acinetobacter baumannii* phage lysin LysAB2 harbor a similar amino acid group, responsible for the passage

of lysin across the outer membrane, and spontaneously lyse Gram-negative cells (Lai *et al.*, 2011; Morita *et al.*, 2001; Orito, Morita, Hori, Unno, & Tanji, 2004). Therefore it is plausible that selected endolysins could genetically be fused to such peptides to target Gram-negative microorganisms. Finally, non-thermal treatments can be used in synergy with endolysins to contribute to food stabilization and shelf-life prolongation. The use of high hydrostatic pressure was shown to permeabilize the cells' outer membrane in order for phage lysins to become active against Gram-negative bacteria (Briers *et al.*, 2008).

General conclusions and future perspectives

In view of the current need to control outbreaks of foodborne pathogens, endolysin-based strategies have revealed to be highly efficient in eliminating microorganisms, meriting their position in the class of new food antimicrobials. Phages outnumber the total amount of bacteria, and therefore a high amount of phages (and with it a reservoir of lysins) on Earth are yet to be discovered. It is expected that for each existing pathogenic bacterium a lysin will be found. However, their application should be carefully studied, as they may not be active in all types of food systems of varied environments. To understand the full potential of these enzymes on modern food enzyme technology, it is important to study real processing conditions on more complex and coordinated enzyme-mediated processes. Also, the more impermeable Gram-negative bacteria deserve particular attention with regard to endolysin application. With *Salmonella* and *Campylobacter* spp being the two most occurring foodborne pathogens, the development of newly isolated, engineered or tailor-made endolysins toward these pathogens will provide new application possibilities.

It is prudent to stress safety issues for the acceptance by food regulatory entities and the final consumer, as far as endolysin application on foodstuff is concerned. Nevertheless, the unique characteristics of endolysins warrant their use as promising novel biocontrol entities in the battle to prevent foodborne infections and to safeguard food quality.

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