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Phage lytic proteins: biotechnological applications beyond clinical antimicrobials.

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Abstract

Most bacteriophages encode two types of cell wall lytic proteins: endolysins (lysins) and virion-associated peptidoglycan hydrolases. Both enzymes have the ability to degrade the peptidoglycan of Gram positive bacteria resulting in cell lysis when they are applied externally. Bacteriophage lytic proteins have a demonstrated potential in treating animal models of infectious diseases. There has also been an increase in the study of these lytic proteins for their application in areas such as food safety, pathogen detection/diagnosis, surfaces disinfection, vaccine development and nanotechnology. This review summarizes the more recent developments, outlines the full potential of these proteins to develop new biotechnological tools and discusses the feasibility of these proposals.

Introduction

Bacteriophages (phages) are viruses that infect bacteria and thus have co-evolved with their bacterial hosts, optimizing their propagation within the cell and the mechanism of their release from within the cell to the environment. Double-stranded DNA phages express virion-associated peptidoglycan hydrolase (VAPGH) proteins that bind to cell surface antigens with high specificity, and disrupt the bacterial cell wall, allowing the phage to inject its DNA into the host cell (Fig. 1) (Moak and Molineux, 2004; Rodríguez-Rubio et al., 2013a). VAPGHs are generally attached to the viral particle contacting the bacterial surface in the first step of the infection process. During the late stages of infection, phage-encoded endolysins (or lysins) accumulate in the bacterial cytoplasm until the assembly of the viral particles is complete. Other bacteriophage-encoded proteins, holins, form pores in the cytoplasmic membrane permitting the translocation of endolysins across the cytoplasmic membrane which degrade the extracellular peptidoglycan (Wang et al., 2000) allowing the cell to osmolyse (holin-endolysin lysis system). Some endolysins have been reported to be secreted by the general bacterial secretion system and remain inactive, membrane-anchored until the membrane potential collapses. This process is triggered by a type of holins, the pinholins, that accumulate in the membrane and form small pores that dissipate the membrane potential (Park et al., 2007).

Despite the fact that the natural mode of action of endolysins is from within, both VAPGHs and endolysins are able to degrade the peptidoglycan of Gram positive bacteria when applied exogenously and hence the interest in their use as alternative antimicrobials (also named enzybiotics) (Nelson et al., 2012; Rodríguez-Rubio et al., 2013a). Actually, the increasing incidence of antibiotic resistant bacteria registered in the past two decades has renewed the interest in the use of phages (phage therapy) and

phage-derived proteins to fight "superbugs" (Sulakvelidze et al., 2001; O'Flaherty et al., 2009). Phage lytic proteins offer some advantages over phage therapy: a wider host spectrum, generally including multiple species from the genus; no potential to transmit virulence factors (e.g. antibiotic resistance genes) and lack of resistance development to phage lysins (Fischetti, 2008; Rodríguez-Rubio et al., 2013b). In fact, since these lytic enzymes encoded by bacteriophages were proposed as new alternatives in the control (Nelson et al., 2012; Shen et al., 2012; Rodríguez-Rubio et al., 2013a), and detection of pathogenic bacteria (Schmelcher and Loessner, 2014) a remarkable number of papers providing information about mode of action, three-dimensional structure, safety, specificity, resistance, immunogenicity, synergy and domain shuffling of phage lytic proteins have been published (reviewed in Schmelcher et al., 2012). For instance, special attention was paid to understand the mode of action and how the presence of more than one catalytic domain could be responsible for the low probability of resistance development (Schuch et al., 2002; Rodríguez-Rubio et al., 2013b).

Structure and enzymatic activity of endolysins and VAPGHs

Depending on their origin, the structure of phage lytic proteins can vary. Most endolysins from phages infecting Gram-positive bacteria have a modular structure with a cell wall binding domain (CBD), usually at the C-terminus and one or two catalytic domains, usually at the N-terminus separated by a short linker (Nelson et al., 2012, Shen et al., 2012). A novel phage lysin, PlySK1249 encoded by a prophage of *Streptococcus dysgalactiae* subsp. *equisimilis* SK1249, has recently been reported to harbor a central CBD surrounded by an N-terminal catalytic domain and a C-terminal catalytic domain (Oechslin et al., 2013). An exception to this modular organization is the structurally unique streptococcal PlyC endolysin, composed of two different gene

products, PlyCA and PlyCB, with a ratio of eight PlyCB subunits for each PlyCA in its active heteromonomer conformation (McGowan et al., 2012). PlyCA subunit harbors the enzymatic activity while the eight PlyCB subunits constitute the CBD for the complete PlyC protein (Nelson et al., 2006). By contrast, most Gram negative phage endolysins have a globular organization with a single catalytic domain (Callewaert et al., 2011) and only some reports indicate a modular organization with one or two CBDs at the N-terminus and one catalytic domain at the C-terminus (Oliveira et al., 2012), which means an inverted molecular orientation compared to endolysins from Gram positive phages.

The Gram negative phage VAPGHs have one catalytic domain, while Gram positive phage VAPGHs have two catalytic domains without a recognizable CBD (Rodríguez -Rubio et al., 2013a).

Catalytic domains are responsible for the cleavage of specific bonds within the bacterial peptidoglycan. The peptidoglycan backbone is a copolymer formed by an alternating sequence of *N*-acetylmuramic acid (MurNAc) and *N*-acetylglucosamine (GlcNAc) linked by β-1,4 bonds. This sugar backbone can have small variations among bacterial species (Schleifer and Kandler, 1972) and is covalently attached to short stem peptides, by an amide bond between the MurNAc and the first amino acid in the chain. The stem peptide is short and formed by an alternating sequence of L and D form amino acids, it is highly conserved in all Gram-negative species but can be highly variable in Gram positive species. In many Gram positive species, the third amino acid residue of the chain is L-lysine. This amino acid attaches via an interpeptide bridge to a terminal D-alanine of another stem peptide. The composition of the interpeptide bridge varies between species. In *Staphylococcus aureus*, for example, the interpeptide bridge is formed by 5 glycines (Navarre et al., 1999) and in *Streptococcus pyogenes* by 2 alanines

(Muñoz et al., 1967). Gram negative species and some Gram positive bacteria, such as *Bacillus* and *Listeria*, have meso-diaminopimelic acid (mDAP) at the third position of the peptide instead of L-lysine. This mDAP residue can directly crosslink to the terminal D-alanine of an opposing stem peptide without forming an interpeptide bridge.

Overall, there are six enzymatic activities associated with phage lytic proteins, grouped into 3 classes:

1. Glycosidases. Include the *N*-acetyl-β-D-glucosaminidase activity, which hydrolyzes the β-1,4 bond between the GlcNAc and the MurNAc of the bacterial peptidoglycan; the *N*-acetyl-β-D-muramidase activity which hydrolyzes the β-1,4 bond between the MurNAc and the GlcNAc and the lytic transglycosylases. By definition, these latter enzymes are not true hydrolase since they do not require water to catalyze the reaction. They are very similar to muramidases as they cleave the β-1,4 bond between the MurNAc and GlcNAc but involving an intramolecular reaction that results in the formation of a 1,6-anhydro ring at the MurNAc residue (Holtje et al., 1975).

2. Amidases. The *N*-acetylmuramoyl-L-alanine amidase activity catalyzes the hydrolysis of the amide bond between MurNAc and the first amino acid of the stem peptide.

3. Endopeptidases. The endopeptidases hydrolyze the bond between two amino acids and include the activities that cleave within the stem peptide of the peptidoglycan, those that cleave bonds within the interpeptide bridge and those that cleave between the stem peptide and the interpeptide bridge.

CBD binds to peptidoglycan ligands or secondary cell-wall polymers like teichoic acids and neutral polysaccharides (Schmelcher et al., 2012; Ganguly et al., 2013) found in the cell wall of the host bacterium. They are involved in positioning the catalytic domain for efficient cleavage of the peptidoglycan and confer some degree of

specificity to the enzyme (Loessner et al., 2002). *Listeria* spp. bacteriophages endolysins are the best studied regarding the binding ligands and specificity of their CBDs (reviewed in Schmelcher et al., 2012). Although CBDs have been shown to be necessary for accurate cell wall recognition and subsequent lytic activity in some peptidoglycan hydrolases, such as lysostaphin (Baba et al., 1996), ALE-1 (Lu et al., 2006), *Listeria monocytogenes* endolysins Ply118 and Ply500 (Loessner et al., 2002) and *Streptococcus pneumoniae* CPL-1 (Pérez-Dorado et al., 2007), there are also numerous reports of C-terminally deleted lysin constructs where the N-terminal lytic domain maintains its activity or it is even higher in the absence of the CBD (Donovan et al., 2006, Cheng et al., 2007, Sass and Bierbaum, 2007, Horgan et al., 2009, Mayer et al., 2011). Net charge of catalytic domains influences on protein activity, thus, catalytic domains with a positive net charge seem to work independently of the presence of a CBD (Low et al., 2011).

The CBDs of some endolysins are directed towards species- or strain-specific cell-wall components that are often essential for viability. For example, amidases PlyG and PlyL selectively bind to secondary cell wall polysaccharides of the bacilli cell wall (Mo et al., 2012; Ganguly et al., 2013), and amidase Pal contains a choline binding module that attaches the enzyme to choline residues present in pneumococcal envelope (García et al., 1988). These conserved catalytic and binding targets have been suggested to contribute to the lack of bacterial resistance development against phage lytic proteins (Fischetti, 2005; Rodriguez-Rubio et al., 2013).

In contrast to endolysins, VAPGHs do not have a recognizable CBD; however the addition of the lysostaphin CBD to the staphylococcal VAPGH HydH5 not only enhanced the staphylolytic activity of the protein but also broadened its host range to different *S. aureus* strains and some *Staphylococcus epidermidis* strains (Rodriguez-

Rubio et al., 2012). HydH5, as a structural protein, is carried to its substrate in the peptidoglycan by the viral particle, which might justify the absence of a CBD. As a soluble protein the scenario is quite different and a CBD was necessary for HydH5 to properly recognize the host and its targeted bond (Rodriguez-Rubio et al., 2012).

There are key differences in protein structure between endolysins and VAPGHs (e.g. the lack of CBD in VAPGHs); however, they are quite similar at the amino acid level suggesting a similar mode of action in their peptidoglycan cleavage mechanisms. Both kinds of proteins hydrolyse specific and highly conserved bonds in the peptidoglycan structure.

As mentioned before, phage lytic proteins are highly refractory to resistant development due to having co-evolved with the host to target conserved bonds in the peptidoglycan (Rodriguez-Rubio et al., 2013). In addition, their modular structure with two catalytic domains could also contribute to the lack of resistance observed since two lytic domains are expected to be higher refractory to resistance development than one domain (Schmelcher et al., 2012; Rodriguez-Rubio et al., 2013b).

State-of-the-art of clinical uses of endolysins

The use of bacteriophage-encoded peptidoglycan hydrolases as new antimicrobials with clinical applications is a promising approach for infectious disease therapy (Table 1). Many studies have been carried out in the past decade to apply lysins to control pathogens systemically and topically on mucosal surfaces and biofilms (Pastagia et al., 2013). The potential of phage lytic enzymes was tested for the first time in 2001 to prevent and treat upper respiratory colonization in mice by group A streptococci (Nelson et al., 2001). After 2 h of the administration of 500 U of lysin PlyC to colonized animals, no remaining bacteria were detected. Subsequent to this work,

multiple studies have demonstrated the efficacy of phage lytic enzymes to control different pneumococcal infections such as pneumonia (Loeffler et al., 2001; Jado et al., 2003; Witzenrath et al., 2009; Doehn et al., 2013), endocarditis (Entenza et al., 2005), otitis media (McCullers et al., 2007), meningitis (Grandgirard et al., 2008), bacteremia (Loeffler et al., 2003) and mucous membranes colonization (Cheng et al., 2005) using murine models of infection. Bactericidal synergism between streptococcal endolysins and antibiotics has also been reported in a murine model of pneumococcal bacteremia. Combination of subtherapeutic doses of Cpl-1, the pneumococcal Cp-1 bacteriophage lysin, with daptomycin significantly increased mice survival (80%) compared with the untreated control (0%) and daptomycin (35%) or Cpl-1 monotherapy (0%) after 7 days of infection (Vouillamoz et al., 2013).

The ability of streptococcal lysins to fight infections has also been tested with other species, e.g., PlyPy, derived from a prophage infecting *S. pyogenes*, was recently reported to rescue mice from systemic bacteremia caused by that pathogen (Lood et al., 2014) and repeated injections within 24h of PlySK1249 lysin significantly protected mice from *Streptococcus agalactiae* bacteremia (Oechslin et al., 2013). Noteworthy is the novel streptococcal lysin with broad lytic activity PlySs2, derived from a *Streptococcus suis* phage, which has been reported to protect mice from a mixed methicillin-resistant *S. aureus* (MRSA) and *S. pyogenes* infection (Gilmer et al., 2013).

Remarkable results have also been achieved in the treatment of *Bacillus anthracis* infections. This pathogen is highly toxic to humans and thus a potential bio-warfare or terrorist agent. Two lysins, PlyG and PlyPH protect mice from death after the infection with *Bacillus* cells (Schuch et al., 2002; Yoong et al., 2006).

Other threatening infections are those caused by MRSA strains against which, bacteriophage-encoded lytic proteins have also been proven effective. In 2007, the

intraperitoneal administration of the endolysin MV-L from phage phiMR11 protected mice against MRSA septic death up to 60 days post infection (Rashel et al., 2007). Similar results were obtained with LysGH15 since an intraperitoneal injection of 50 μg of the protein administered 1 h after MRSA infection was sufficient to protect mice from death (Gu et al., 2011). Recently, an intravenous injection of a pre-formulation containing recombinant endolysin SAL-1 prolonged the viability of mice and significantly reduced MRSA counts in the bloodstream and splenic tissue (Jun et al., 2013). Another staphylococcal endolysin able to protect mice from a staphylococcal bacteremia is endolysin P-27/HP which is able to reduce by 99.9% the presence of *S. aureus* in mice spleens (Gupta and Prasad, 2011). Chimeric proteins have also been tested against staphylococcal infections. The chimeric lysin, ClyS, was able to reduce by 2-log the MRSA numbers after the administration of one dose in a mouse nasal colonization model, to protect animals against death by MRSA in a mouse septicemia model and to eradicate skin infections more efficiently than the standard topical antibiotic mupirocin (Daniel et al., 2010; Pastagia et al., 2011). Another bacteriophage-derived chimeric protein, P128, was able to reduce by 2-log an initial MRSA colonization of ~10^9 CFU using 100 μg of the protein twice a day for 3 days in a rat nasal colonization model (Paul et al., 2011). Truncated endolysins are also potential antistaphylococcal agents as proved by the peptidase domain from endolysin LysK ($CHAP_k$) which is effective in the elimination of *S. aureus* from mice nares as well as a decolonization agent for the removal of this pathogen from the surface of mammalian skin (Fenton et al., 2010; Fenton et al., 2013). The application of $CHAP_k$ as a spray removed 99% of *S. aureus* from porcine skin in 30 min (Fenton et al., 2013). Anti-staphylococcal endolysins have been combined with antibiotics to treat MRSA bacteremia showing superior efficacy to antibiotic therapy alone. This is the case of

endolysin CF-301, also referred to as PlySs2 (Gilmer et al., 2013), which was combined with vancomycin or daptomycin increasing mice survival significantly ($P<0.0001$) when compared to antibiotics alone (Schuch et al., 2014).

Endolysins are largely ineffective against Gram negative bacteria, the primary hurdle being the inability of these proteins to cross the bacterial outer membrane. To solve this problem several approaches have been reported including the use of membrane-disaggregate agents (Callewaert et al., 2011) and fusion of lysins to protein domains able to cross the outer membrane (Lukacik et al., 2012). More recently Briers and colleagues have developed new endolysin-based antibacterials, named artilysins, which combine a phage endolysin with a targeting peptide that transports the endolysin through the outer membrane of Gram negative bacteria (Briers et al., 2014a). Thus, Art-175 is able to pass the outer membrane and kill *Pseudomonas aeruginosa*, including multidrug-resistant strains and persisters, in a rapid and efficient fashion (Briers et al., 2014b). Despite the fact that a notable number of endolysins against Gram negative bacteria have been studied no clinical assays in animals or in humans have been reported.

Overall, bacteriophage-encoded lytic proteins appear to be useful and effective as therapeutics against multi-drug resistant Gram positive pathogens. These phage-encoded lytic enzymes could also be used as disinfectants of medical devices (orthopaedic prostheses, indwelling catheters, etc.) to prevent colonization and avoid post-surgery infections. However, before extending their use in human therapy it is necessary to perform clinical trials to study the effects of these proteins on human systems, including a determination of the immune response to these protein agents.

Main biotechnological applications

Phage lytic protein applications in different areas are outlined below and summarized in Figure 2.

1. Food safety. The continuous increase in foodborne diseases caused by pathogens such as *Salmonella*, *Campylobacter*, *Escherichia coli* and *Listeria* (EFSA Journal, 2012) has promoted the research of new technologies to inactivate bacteria in food. An essential requirement that should be met by any of these new approaches is that it must be safe for humans, animals and the environment while keeping the nutritional value and the organoleptic properties of the final product undisturbed. Moreover, contaminating bacteria can gain access to food during all stages of production (slaughtering, milking, fermentation, processing, storage, packaging) and these new technologies have to be applied throughout the complete food chain ("farm to fork"). Bacteriophages have been proposed as alternatives to antibiotics in animal health, as biopreservatives in food and as tools for detecting pathogenic bacteria throughout the food chain (García et al., 2008). The approval of bacteriophages as food processing aid in USA [LISTEX™ and SALMONELEX™, Micreos Food Safety (http://www.micreos.com); ListShield™, EcoShield™, SalmoFresh™, Intralytix (http://www.intralytix.com)] confirms the feasibility of this application. In this context, phage lytic proteins have remarkable advantages for food preservation; their narrow spectrum of activity allows them to target pathogenic or spoilage bacteria without disturbing the commensal microbiota when they are used to reduce colonization in livestock. Moreover, they are not active against starter cultures when they are applied as preservatives to extend the shelf life of fermented products (García et al., 2010). However, their inefficiency against Gram negative bacteria is a disadvantage for the use of phage-encoded lytic proteins as biopreservatives against those pathogens.

In agriculture, protection against the phytopathogen *Erwinia amylovora* was obtained in transgenic potatoes synthesizing the endolysin from the *Escherichia coli* phage T4 (Düring et al., 1993). Moreover, direct application of the recombinant endolysin from *E. amylovora* phage Ea1h on surfaces of immature pears resulted in a delay of necrosis produced by this pathogen (Kim et al., 2004). Other endolysins like CMP1 and CN77, from *Clavibacter michiganensis* phages, have been proposed to control this plant pathogen without affecting other bacteria in the soil (Wittmann et al., 2010). Regarding animal production, transgenic cows expressing endolysins have been proposed to reduce mastitis and *S. aureus* milk contamination (Donovan et al., 2006). In fact, chimeric phage lysins were tested in combination with lysostaphin in a mouse model of mastitis where they reduced *S. aureus* counts, gland weights and intramammary tumor necrosis factor alpha (Schmelcher et al., 2012). In food processing, pathogen biocontrol by endolysins has been approached by addition of these proteins as biopreservatives and by expressing endolysin genes in starter or protective cultures. The purified staphylococcal endolysin LysH5 killed *S. aureus* in pasteurized milk within 4 h (Obeso et al., 2008) and its activity was enhanced by a synergistic effect with nisin (García et al., 2010). Furthermore, the VAPGH-derived fusion protein CHAPSH3 (CHAP domain from Hyd5 fused to the SH3 domain from lysostaphin), from staphylococcal phage vB_SauS-phiIPLA88, was shown to be active in raw milk and retain staphylolytic activity after pasteurization and cold-storage, which could be useful in dairy product manufacturing (Rodríguez-Rubio et al., 2013c). In soya milk, *Listeria monocytogenes* contamination was reduced at refrigeration temperature by addition of endolysin LysZ5 (Zhang et al., 2012). Other lysins such as the streptococcal lysins B30 (Donovan et al., 2006) and Ply700 (Celia et al., 2008) and the chimeric streptococcal–staphylococcal constructs λ SA2E-Lyso-SH3b and λ SA2E-LysK-SH3b

(endopeptidase domain from streptococcal LambdaSa2 endolysin fused to lysostaphin or LysK SH3b domain, respectively) have also been reported to be active in milk (Schmelcher et al., 2012). In addition, endolysin ctp1l was proposed as an alternative to avoid late blowing in cheese production by *Clostridium tyrobutyricum* (Mayer et al., 2010). It is tantalizing to consider that recombinant bacteria expressing endolysins might exert protective effects in food fermentations (Gaeng et al., 2000). However, the listerial phage endolysin Ply511 expressed in an active form in lactic acid bacteria was unable to significantly inhibit *L. monocytogenes* growth (Turner et al., 2007). The staphylolytic endolysin LysH5 that is active in milk (Obeso et al., 2008) was expressed and secreted from *L. lactis* using the signal peptide of bacteriocin lactococcin 972 but the protective activity in milk has not yet been assessed (Rodríguez-Rubio et al., 2012). Although it is known that endolysins are active in different food matrices, additional information is needed as to the effectiveness of these proteins after oral administration in humans.

2. Pathogen detection. Rapid and effective detection of pathogens is very important for the proper treatment and prevention of disease. Regarding the use of phage proteins to detect pathogens, most of studies have focused on the detection of foodborne bacteria.

In 2007, Kretzer et al. developed a system to detect and immobilize cells of *L. monocytogenes* on magnetic beads coated with CBDs from different *Listeria* phages endolysins, enabling detection rates of >90% which is superior to the standard plating procedure in terms of both sensitivity and time required. Subsequently, Schmelcher et al. (2010) used this method to recover *Listeria* cells from inoculated milk and cheese, and were able to differentiate serotypes after incubation with CBDs fused to different fluorescent proteins. Tolba et al. (2012) also developed a biosensor for a rapid and specific detection of *Listeria* cells using endolysins CBDs immobilized on a gold screen

printed electrode and subsequent electrochemical impedance spectroscopy. The limit of detection was 1.1×10^4 and 10^5 CFU/ml in pure culture of *Listeria innocua serovar 6b* and 2% artificially contaminated milk, respectively (Tolba et al., 2012). Other methods based on endolysins have been developed to detect *B. anthracis* (Fujinami et al., 2007, Sainathrao et al., 2009). Using the C-terminal region of γ-phage lysin protein (PlyG), Fujinamii et al., (2007) developed a bioprobe to detect *B. anthracis* with a membrane direct blot assay which turned out to be simpler and less expensive than other genetic tools such as PCR, or immunological methods using specific antibodies. Later, Sainathrao et al. (2009) demonstrated that a 10-amino acids motif from the C-terminal region of PlyG coupled with fluorescent Qdot-nanocrystals is sufficient to specifically detect *B. anthracis*.

3. Surface disinfection. One of the most important challenges in clinical environments and in food industry is the presence of bacterial biofilms on medical devices and industry surfaces (Otto, 2008; Gutiérrez et al., 2012). Bacteria embedded in biofilms are considerably less susceptible to antibiotics and disinfectants than their planktonic counterparts, due to both a reduced growth rate and limited access of those compounds to bacteria in a biofilm (Davies, 2003). Reduced susceptibility to antibiotics of some bacteria within the biofilm which showed a more recalcitrant phenotypic state (persister cells) have been also implicated in recurrent infections (Lewis, 2007). Problems associated with biofilms could be overcome by using endolysins and VAPGHs and they have been tested in both clinical and food production environments. Staphylococcal phage endolysins and truncated derivative proteins have been tested against *S. aureus* and *S. epidermidis* biofilms. The recombinant endolysins of phages phi11 and SAP-2 were able to remove *S. aureus* biofilms formed on polystyrene surfaces (Sass and Bierbaum, 2007; Son et al., 2010). In addition, bacteriophage K derived peptidase,

$CHAP_k$, successfully prevented and removed *S. aureus* biofilms formed on artificial surfaces (Fenton et al., 2013). Furthermore, endolysin LysH5 was almost as effective against *S. aureus* as *S. epidermidis* biofilms and no resistant cells were observed after treatment with this protein (Gutiérrez et al., unpublished). *S. suis* is an important animal pathogen that is also a zoonotic agent infecting humans. A phage endolysin, LysSMP led to more than 80% reduction of biofilms formed by these bacteria (Meng et al., 2011). Moreover, other phage endolysins, Cpl-1 and Cpl-7, were very efficient in destroying biofilms formed by *S. pneumoniae* and the closely related species *Streptococcus pseudoneumoniae* and *Streptococcus oralis* (Domenech et al., 2011). Recently, Shen et al., (2013) demonstrated that endolysin PlyC directly lyses *S. pyogenes* cells within the biofilm matrix due to its ability to diffuse through the extracellular material.

4. Vaccine development. Vaccination, stimulating the immune system to increase the adaptive immunity to a pathogen by the administration of antigenic material, represents one of the greatest public health triumphs. Viral nanoparticles (VNPs) have been an active area of research as delivery platforms for protein and peptide-based vaccines. Bacteriophage-derived nanoparticles, however, have attracted much interest since foreign peptides or proteins can be displayed on the surface of phages as fusion proteins. Thus, phage-displayed peptides or proteins have been shown to be functionally and immunologically active, what makes them suitable for vaccine development (Hamzeh-Mivehroud et al., 2013). In the search of live vaccine delivery systems, lysins can also be used as anchor to display heterologous proteins on the surfaces of bacteria. For example, CBDs can be fuse to other proteins (such as antigens) in order to display them at the bacterial surface, conserving both their conformation and activity (Loessner et al., 2002). Lactic acid bacteria (LAB) have been used to develop surface display

systems based on phage endolysins (Hu et al., 2010, Ribelles et al., 2013) in large part due to their GRAS status (generally recognized as safe), which makes them more readily accepted in medical and food applications than other bacteria. Regarding the application of these systems, recently, Ribelles et al. (2013) demonstrated that the exogenous addition of the E7 antigen, from human papillomavirus type-16 (HPV-16), when anchored on the surface of LAB by the CBD of *Lactobacillus casei* A2 phage endolysin, protected mice against a HPV-16-challenge and the resultant tumors. Over 60% of the mice remained tumor-free throughout the test period (35 days) after being immunized via intranasal vaccination with LAB displaying E7 antigen; the rest of mice presented tumors 10-fold smaller than the ones measured in non-treated mice. Thus, surface display of endolysins CBDs is an interesting system to further direct therapeutic proteins to the surface of otherwise non-genetically modified bacteria in a safe and low-cost way.

Endolysins have also been used to produce empty 'ghost' bacteria to be developed into vaccines. These bacterial 'ghosts' are produced by the controlled expression of phage PhiX174 lysis gene *E*. E-mediated lysis of bacteria outcomes in the formation of empty bacterial cell envelopes with essentially the same cell surface composition as their living counterparts (Eko et al., 1999; Haidinger et al., 2003). These ghost bacteria are able to induce a strong immune response and to protect against infection. Thus, mucosal immunization with 'ghost' cells protects over 93% of mice against *E. coli* O157:H7 and *Helicobacter pylori* (Mayr et al., 2005; Panthel et al., 2003). Ghost-bacteria oral vaccines have also been used with promising results to prevent the infection of fish with *Aeromonas hydrophila* and *Flavobacterium columnare* (Tu et al., 2010; Zhu et al., 2012) and protected chicken against *Salmonella* Enteritidis (Peng et al., 2011). However, the development of genetically inactivated

bacterial vaccines often requires the use of plasmid encoded antibiotic resistance gene markers, whose presence can increase the risk of horizontal spread of those resistance genes. In this context, Jawale and Lee (2013) developed an immunogenic *Salmonella* Enteritidis ghost using an antibiotic resistance marker free plasmid carrying the bacteriophage PhiX174 lysis system. This *S.* Enteritidis ghost was evaluated in chickens by prime-boost vaccination using a combination of oral and intramuscular routes. After a *S.* Enteritidis challenge, all immunized animals showed fewer gross lesions and decreased bacterial recovery from organs in comparison with the non-immunized control group (Jawale and Lee, 2013).

5. Nanotechnology. Among all the fields that enclose the nanotechnology, its relevance in the food sector is a relatively recent event compared with its use in medicine and pharmaceutics. In fact, many researchers in bionanoscience and bionanotechnology were conducted, and phage-based nanomaterials have been described for biomedical applications (Farr et al., 2013). Phage lytic enzyme Ply500 was used for surface-based applications to prevent contact-based bacterial infections. For biotechnological processes, endolysins should be immobilized onto surfaces, with long shelf-lives and activity. The immobilization of Ply500 onto FDA approved nanoscale silica particles (Ply500-SNP), improved its stability at both 4ºC and 25ºC, and a step forward was achieved when incorporating this Ply500-SNP to a poly-hydroxyethyl methacrylate film which was able to completely remove listeria cells on contact at 4ºC (Solanki et al., 2013). This might find application for endolysins as potential anti-bacteria coatings of cold storage or food processing equipment. Moreover, starch conjugates with endolysins may find further application in antimicrobial packaging systems like spraying/coating on food before packaging or incorporation into packaging materials

(Solanki et al., 2013). Nanotechnology opens new avenues for the application of endolysins and VAPGHs in different sectors to improve human health, and food safety.

Other applications

Bacteriophage-encoded lytic proteins can also have other biotechnological applications different from those mentioned above. Phage lysins can also be useful to identify novel targets for antimicrobials development. This is the case of *B. anthracis*-specific endolysin PlyG which was used to identify an enzyme involved in the synthesis of its receptor and bacterial growth (Schuch et al., 2013). By synthesizing a specific inhibitor of this enzyme, named epimerox, authors were able to obtain a potent inhibitor of *B. anthracis* growth *in vitro* and *in vivo* in mice challenged with lethal doses of the pathogen (Schuch et al., 2013).

The phage holin–endolysin lysis system can be used in the microbial production of bio-based chemicals to disrupt cells and facilitated downstream processing. This approach is economically more feasible and easier to control than traditional methods of cell disruption (reviewed by Gao et al., 2013). Furthermore, phage endolysins have been used for DNA, RNA and protein release from Gram-positive bacteria such as *Listeria* (Loessner et al., 1995) or *S. pyogenes* (Köller et al., 2008) and for the development of auto-inducible or heat-inducible *E. coli* lysis system to facilitate protein expression (Zhang et al., 2009; Carnes et al., 2009).

Two recent applications of bacteriophage-encoded lytic enzymes is the control of bacterial growth in biofermentation and in diagnostic testing. Contamination of fermentation cultures by LAB reduces yields of ethanol, weakening the economics of biofuel production. Roach et al. (2013) demonstrated that the streptococcal phage LambdaSa2 endolysin and several *Lactobacillus* bacteriophage endolysins were able to

lyse lactobacilli, staphylococci or streptococci strains under fermentation conditions. In addition, LambdaSa2 endolysin and one of the *Lactobacillus* bacteriophage endolysins were able to reduce both *L. fermentum* and *L. reuteri* contaminants in mock fermentations of corn fiber hydrolysates (Roach et al., 2013). Another example of the control of bacterial growth using phage endolysins was proposed by Subramanyam et al. (2013). These authors used a pool of three endolysins to control the overgrowth of the normal microbiota in processed sputum samples in order to properly detect *Mycobacterium tuberculosis.* The rate of growth of normal microbiota for the detection method using the phage lysin pool was 9.3% compared with untreated samples (Subramanyam et al., 2013). Similar results were obtained when antibiotics were used to decontaminate sputum samples (Subramanyam et al., 2011); however, antibiotics also reduce the growth rate of mycobacteria. In this context, endolysins demonstrated to be as effective as antibiotics without inhibiting *M. tuberculosis* growth (Subramanyam et al., 2013).

Perspectives and conclusions

Endolysins and VAPGHs have repeatedly shown their antimicrobial potential to fight against numerous pathogens, being a feasible alternative to antibiotics in the treatment of infectious diseases. Results are so promising that new approaches are being investigated to exploit this antimicrobial potential in other fields such as food safety, surface decontamination and nanotechnology. However, not only their antimicrobial activities make these proteins attractive but also the specificity of their CBDs can be exploited as powerful tools for bacterial detection and antigen presentation. Considering that bacteriophages are the most abundant live entities, phage lytic proteins are structurally and functionally diverse which along with current protein engineering tools

opens a promising future with broad potential for these proteins. Nevertheless, the prospects of phage lytic proteins as biotechnological tools have several hurdles to overcome. For instance, in a food safety context, the approval of phage-derived proteins as authorized biopreservatives, the production costs and consumer acceptance seem to be the primary obstacles. Although no adverse effects were observed after mucosal or systemic administration of phage lytic proteins (Fischetti, 2008), further research is needed on their cytotoxicity and immunogenicity in order to confirm their safety for oral administration as food additives. It is known that endolysins are able to generate an antibody response but neutralization of the protein was not observed (Jado et al., 2003). Another issue to overcome for the pharmacological or biotechnological use of endolysins, especially for disinfection of food industry or healthcare environments, is the thermolabile nature of these enzymes. For example, it has been reported that *S. aureus* endolysin LysK, *S. pneumoniae* endolysins Cpl-1 and Pal and *S. pyogenes* endolysin PlyC lose activity at 42°C, 43.5°C, 50.2°C and 45ºC respectively (Filatova et al., 2010; Sanz et al., 1993; Varea et al., 2004; Heselpoth et al., 2012). Another example is *S. aureus* endolysin LysH5 which is active in milk but has no activity after 30 min at 63ºC (Obeso et al., 2008) conditions used in milk pasteurization. Thus, increase thermostability of phage endolysins by protein engineering can be an important approach for future applications of these enzymes. Regarding nanotechnological applications, phage lytic proteins have a great potential for development of new materials because they provide a non-toxic approach to eliminate pathogens through bactericidal surfaces in food and healthcare environments. In addition, the very strong binding affinity of their CBDs for the bacterial cell wall ligands makes these domains a perfect alternative to antibodies, which are more laborious to obtain, for biosensor development.

The potential of phage lytic proteins is diverse. There are several companies working on phage therapy (Ampliphi, Phage Biotech, Intralytix, Gangagen), who have paved the way for phage applications in both human and animal health, and food safety. It is a natural progression to expect that the similarly diverse properties and high potential of phage lytic proteins as antimicrobials and biotechnological tools will encourage the exploitation of these unique proteins. In fact, nowadays Micreos Human Health already has on the market several endolysin-based products under the GLADSKIN™ brand (www.gladskin.com) for people with acne, eczema and rosacea, and ContraFect Corporation (Schuch et al., 2014) is testing, in Phase I clinical trials, phage lytic proteins for treatment of life-threatening, drug-resistant infectious diseases.

Declaration of interest

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Tables

Table 1. Potential clinical uses of bacteriophage-encoded peptidoglycan hydrolases

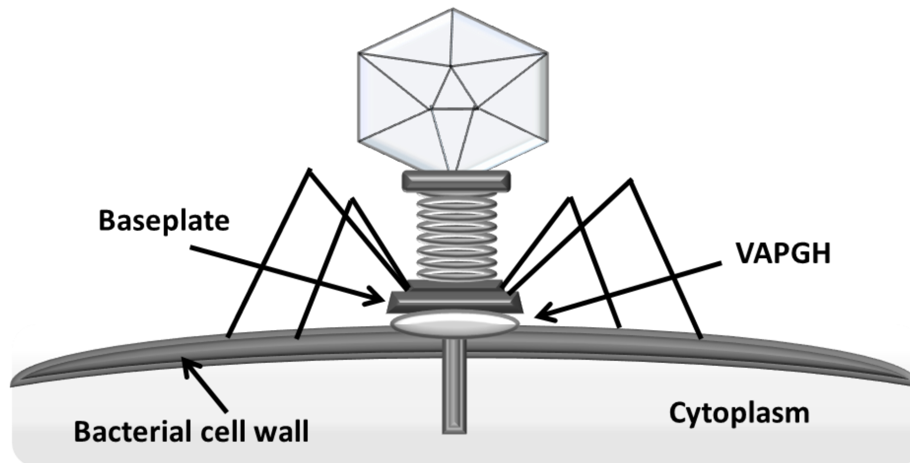
ENDOLYSIN	TREAT OR PREVENT	CAUSED BY	REFERENCE
PlyC	Upper respiratory colonization	Group A streptococci	Nelson et al., 2001
Pal	Nasopharyngeal colonization and bacteraemia	*S. pneumoniae*	Loeffler et al., 2001, Jado et al., 2003
Cpl-1	Bacteremia, endocarditis, meningitis, pneumonia, otitis media	*S. pneumoniae*	Jado et al., 2003, Loeffler et al., 2003, Entenza et al., 2005, McCullers et al., 2007, Grandgirard et al., 2008; Witzenrath et al., 2009, Doehn et al., 2013, Vouillamoz et al., 2013
PlyPy	Bacteremia	*S. pyogenes*	Lood et al., 2014
PlySK1249	Bacteremia	*S. agalactiae*	Oechslin et al., 2013
PlySs2 (CF-301)	Mixed infections and bacteremia	MRSA, *S. pyogenes*	Gilmer et al., 2013, Schuch et al., 2014
PlyGBS	Vagina and oropharynx colonization	Group B streptococci	Cheng et al., 2005
PlyG	Infections	*B. anthracis*	Schuch et al., 2002
PlyPH	Infections	*B. anthracis*	Yoong et al., 2006
MV-L	Septic death	MRSA	Rashel et al., 2007
LysGH15	Septic death	MRSA	Gu et al., 2011
SAL-1	Septic death	MRSA	Jun et al., 2013
P-27/HP	Bacteremia	*S. aureus*	Gupta and Prasad, 2011
ClyS	Nasal colonization, skin infections and septicemia	MRSA	Daniel et al., 2010; Pastagia et al., 2011
P128	Nasal colonization	MRSA	Paul et al., 2011
$CHAP_k$	Nasal and skin colonization	*S. aureus*	Fenton et al., 2010, 2013

Figures

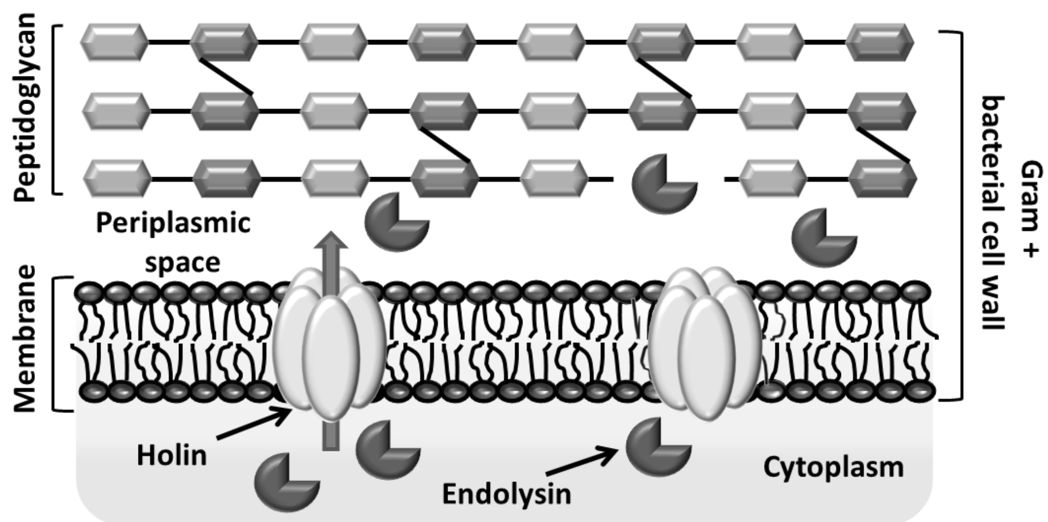
Figure 1. Mode of action of phage lytic proteins. A) Location of VAPGH protein at the phage particle and its role in the infection process. B) Structure of Gram positive bacteria cell wall and role of the endolysin protein. Light grey hexagon: *N*-acetylglucosamine (GlcNAc); Dark grey hexagon: *N*-acetylmuramic acid (MurNAc).

Figure 2. Main biotechnological applications of phage lytic proteins. Modular structure of these proteins (EAD= enzymatically active domain, CBD= cell binding domain) is indicated. Pathogen detection and vaccines development have been mainly developed by using the CBD of lytic proteins, while for food safety, surface disinfection and nanotechnology purposes complete proteins were used. LAB: lactic acid bacteria.

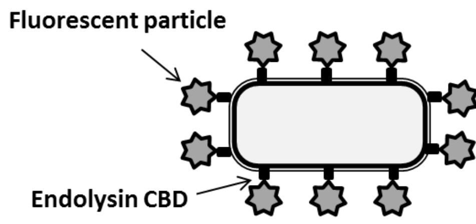
A)



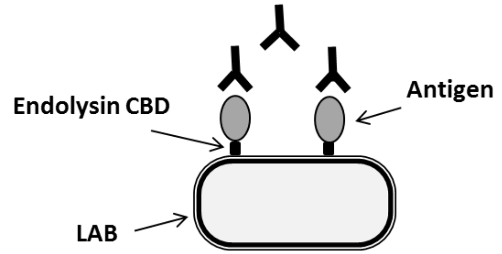
B)

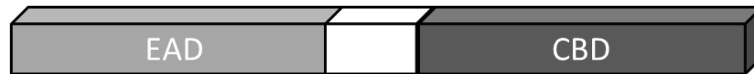


PATHOGEN DETECTION

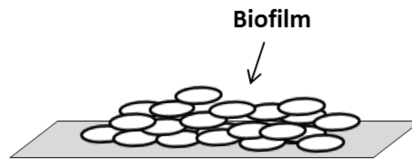


VACCINES DEVELOPMENT

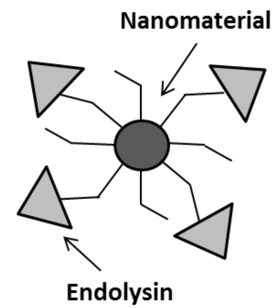




FOOD SAFETY



SURFACE DISINFECTION



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