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BACTERIOPHAGE ENDOLYSINS AND THEIR USE IN BIOTECHNOLOGICAL PROCESSES

Lenka Tišáková[1], Andrej Godány[1,2]*

Address(es): RNDr. Lenka Tišáková,
[1] Institute of Molecular Biology Slovak Academy of Sciences, Department of Genomics and Biotechnology, Dúbravská cesta 21, SK-845 51, Bratislava, Slovakia, phone number: +421 2 59307432.
[2] University of Ss. Cyril and Methodius in Trnava, Faculty of Natural Sciences, Department of Biotechnology, J. Herdu 2, SK-917 01, Trnava, Slovakia.

*Corresponding author: lenka.tisakova@savba.sk

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ABSTRACT

Bacteriophage endolysins are peptidoglycan hydrolases, produced in the lytic system of bacteriophage in order to lyse host peptidoglycan from within and release virions into the environment. Phages infecting Gram-positive bacteria express endolysin genes with the characteristic modular structure, consisting of at least two functional domains: N-terminal enzymatically active domain (EAD) and C-terminal cell wall binding domain (CBD). CBDs specifically recognize ligands and bind to the bacterial cell wall, whereas EAD catalyze lysis of the peptidoglycan bonds. The reveal of endolysin modular structure leads to new opportunities for domain swapping, construction of chimeras and production of specifically engineered recombinant endolysins and their functional domains with the diverse biotechnological applications from without, such as in detection, elimination and biocontrol of pathogens, or as anti-bacterials in experimental therapy.

Keywords: bacteriophage endolysins, EAD, CBD, applications

INTRODUCTION

Most of the tailed phages achieve correctly timed lysis by the consecutive use of two phage-encoded lysis proteins - **endolysin** and **holin (Young *et al.*, 2000; Loessner, 2005)**. All dsDNA phages produce a soluble, muralytic enzyme known as an endolysin. To degrade the cell wall, endolysins require a second lysis factor, a holin. Host cell lysis is mostly strictly regulated and exactly timed with the help of a holin. Holins are small hydrophobic proteins that are inserted into the cytoplasmatic membrane. At a genetically determined time in the terminal stage of the lytic cycle and upon a critical holin effector concentration and partial depolarization of the membrane, the holin monomers instantly assemble into oligomers and form membrane lesions "holes" through which the endolysins can then pass and lyse the bacterial cell wall **(Vukov *et al.*, 2003; Loessner, 2005; Fenton *et al.*, 2010)**. Breach of the peptidoglycan (PG) layer results in osmotic lysis and cell death of the bacterium, thus enabling liberation of progeny virions **(Schmelcher *et al.*, 2012)**. Most known endolysins lack a secretory signal sequence and depend entirely on the cognate holin, which somehow permeabilizes the membrane, and is required for the endolysin to gain access to the murein for release to the PG **(Loessner *et al.*, 2002; Loessner, 2005)**.
In most dsDNA phages the holin and endolysin genes cluster together, respectively, into the so called "lysis cassette" as part of the late transcribed genes (Fig. 1), although deviations from this spatial and temporal organization are found both in Gram-negative and Gram-positive systems. In addition to this basic lytic function, other phage-encoded proteins may work as auxiliary lysis factors **(Young *et al.*, 2000)**. In general, phage-encoded endolysins are easily identified by simple analysis of phage genomic sequences, due to the relatively high amino acid conservation observed within their catalytic domains **(São-José *et al.*, 2007).**
Bacteriophage endolysins are dsDNA bacteriophage-encoded peptidoglycan hydrolases (PGHs) which are synthesized in phage-infected bacterial cells during the late phase of gene expression at the end of multiplication cycle (Loessner, 2005). They are similar in structure and function to bacterial auto- and exo-lysins **(López *et al.*, 1997; Shen *et al.*, 2012)** and also closely related to the similar family of mammalian PG recognition proteins **(Low *et al.*, 2011)**. Numerous studies have investigated the specificity of endolysins by assaying the cleavage sites on purified PG **(Loessner *et al.*, 1998; Navarre *et al.*, 1999; Pritchard *et al.*, 2004; Dhalluin *et al.*, 2005; Fukushima *et al.*, 2007; 2008; Mayer *et al.* 2008; 2011)**.

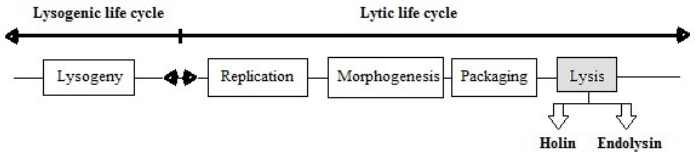

Figure 1 Bacteriophage genome architecture. The diagram represents a typical template bacteriophage genome with genes involved in specific stages in phage development being grouped into modules (boxes). The specific genes/DNA sequences for holin and endolysin within the lysis module are indicated (bold text) (according to **McGrath *et al.*, 2004**)

ENDOLYSIN MODULAR ARCHITECTURE

Generally, the structure of bacteriophage endolysins differ between those enzymes targeting Gram-positive and Gram-negative bacteria, reflecting the differences in the cell wall architecture between these major bacterial groups. Endolysins from a Gram-positive background have evolved to utilize a modular design. This is achieved by the combination of at least two distinct polypeptide modules (separated functional domains) that are dedicated two basic functions: substrate recognition and enzymatic hydrolysis, as depicted in Fig. 2. The N-terminal **enzymatically active domain(s) = EAD** generally harbor the catalytic activity (cleaving the specific bonds within the bacterial PG), whereas the C-terminal **cell wall binding domain(s) = CBDs** direct the enzymes to their substrates **(Loessner *et al.*, 2002; Fischetti, 2005; Loessner, 2005)** and 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 **(Loessner *et al.*, 2002)**. Most of the endolysins studied to date are composed of at least two clearly separated functional domains **(Loessner, 2005; Borysowski *et al.*, 2006; Hermoso *et al.*, 2007; Fenton *et al.*, 2010; Fischetti, 2010; Schmelcher *et al.*, 2012)**. However, endolysin structures are not necessary limited to only two modules **(Diaz *et al.*, 1990; Garcia *et al.*, 1990)**, and the different architectures and domain orientations found in public databases are various **(Nelson *et al.*, 2012)**.

Figure 2 Schematic representation of the bacteriophage endolysin infecting Gram-positive bacterial strains, illustrating its two-domain modular architecture. N-terminal catalytic and C-terminal binding domain are separated by a flexible linker region

It is important to note that, with respect to Gram-positive cells, endolysins can also act as exolysins, in most cases, accessible from without. When exogenously applied to Gram-positive cells, endolysins can digest the PG and cause lysis from without due to absence of an outer membrane (OM) in these bacteria **(Loessner, 2005)**. This is not the case for Gram-negative cells, in which the presence of the OM effectively prevents access by hydrophilic lytic enzymes. However, when the lipopolysaccharide layer is disrupted (by EDTA, detergents, etc.), cells immediately become more sensitive to external PGHs. In general, lysin-treated bacteria examined by thin section electron microscopy revealed that endolysins cause their lethal effects by forming holes in the cell wall through PG digestion. It is known that any disruption of the cell wall integrity will result in the extrusion of the cytoplasmatic membrane and ultimate hypotonic lysis **(Loessner *et al.*, 2002)**. Moreover, catalytically, a single enzyme molecule should be sufficient to cleave an adequate number of bonds to kill a bacterium, however, it is uncertain at this time whether this theoretical limit is possible **(Loessner, 2005; Fischetti, 2010)**. **Loessner *et al.* (2002)** demonstrated this idea on a listeria phage enzyme that showed a binding affinity approaching that of an IgG molecule for its cell wall substrate. This suggested that phage lytic enzymes are one-use enzymes, likely requiring several molecules attacking a local region to sufficiently weaken the cell wall **(Loessner *et al.*, 2002, Fischetti, 2010)**.
There is increasing evidence that many bacteriophage endolysins display a structure that reflects a combination of individual functional domains, or modules (CBDs and EADs). In some cases, the modular architecture of bacteriophage endolysins could be experimentally demonstrated by deletion analyses or construction of chimerical proteins **(Oshida *et al.*, 1995; Baba and Schneewind, 1996; Loessner *et al.*, 1998; 2002; Navarre *et al.*, 1999; Morita *et al.*, 2001)**. Comparing the sequences of lytic enzymes of the same enzyme class revealed high sequence homology within the N-terminal catalytic region and very little homology with the C-terminal cell wall binding region **(Loessner *et al.*, 2002)**. Efficient cleavage requires that the binding domain binds to its cell wall substrate, offering some degree of specificity to the enzyme since these substrates are only found in enzyme-sensitive bacterial hosts **(Loessner *et al.*, 2002; Hermoso *et al.*, 2003; Fischetti, 2010)**. Because of this specificity, enzymes that are released after cell lysis had a good chance of killing potential bacterial hosts in the vicinity of the released phage progeny. Thus, it is believed that the endolysins have evolved to bind to their CBDs at a high affinity **(Loessner *et al.*, 2002)** to limit the release of free enzyme **(Fischetti, 2010)**. Finally, because of their domain structure, it seemed convenient that different enzyme domains could be swapped resulting in endolysins with different bacterial and catalytic specificities **(Garcia *et al.*, 1990)**.

Enzymatically active domains of endolysins

The EAD embraces the lytic mechanism of an endolysin, catalyzing the breakdown of the PG. Due to the moderately conserved overall structure of the PG, there are limited types of covalent bonds available for cleavage by endolysins and other PGHs. Considering this fact, endolysins can be classified to at least four different groups depending on the specific bond of the PG attacked by the EAD: (i) **glycosidase**, (ii) **endopeptidase**, (iii) a specific **amidohydrolase**, and (iv) lytic **transglycosylase (Borysowski *et al.*, 2006; Loessner, 2005; Fenton *et al.*, 2010; Nelson *et al.*, 2012)**, although there are some differences in terminology among authors. Furthermore, databases contain terms specifying families of conserved EADs, such as the CHAP domain **(Bateman and Rawlings, 2003; Zou and Hou, 2010)**, which refers to the molecular mechanism by which the cleavage occurs rather than the specific bond cleaved. While assignment of cleavage specificity to EADs of newly discovered endolysins through bioinformatics analysis (i.e. based on sequence homology with other domains) is the most commonly used method, it can yield incorrect results that can readily be propagated within the databases. In comparison, there are numerous experimental ways to clearly determine an enzyme´s catalytic specificity, including e. g. various classical biochemical methods, N-terminal sequencing of digestion products and mass spectrometry **(Nelson *et al.*, 2012)**.
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. Knowing this fact, amidases and muramidases are not only the most commonly found enzymes **(Loessner, 2005)**, but also are among the most universal ones, targeting highly conserved bonds in the PG. 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 **(Vollmer, 2008)**. Moreover, since 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 **(Low *et al.*, 2011)**. As **Loessner (2005)** reviewed, the catalytic domains of some particular streptococcal endolysins require activation from their low-activity (E-form) to a fully active C-form state. This conversion occurs upon contact with a cell wall ligand, which ultimately leads to changes in the catalytic region **(Saiz *et al.*, 2002; Varea *et al.*, 2004)** that appear to be reversible **(Romero *et al.*, 2004)**. However, conformational alternations were excluded by crystal structures **(Loessner, 2005)**.
Interestingly, an EAD may possess the ability to bind to the bacterial cell wall surface independently of its respective CBD and contain features conferring inherent cell wall specificity. On the one hand, some endolysins have been reported to require a CBD for full lytic activity from without **(Sanz *et al.*, 1992; Donovan *et al.*, 2006; Korndörfer *et al.*, 2006; Porter *et al.*, 2007)**, while others are equally or even more active when the binding domain is deleted **(Gaeng *et al.*, 2000; Low *et al.*, 2005; Cheng and Fischetti, 2007; Mayer *et al.*, 2011)**.

Cell wall binding domains of endolysins

The CBD gives specificity to an endolysin for certain cell wall types by recognizing and binding non-covalently to **ligand** molecules within the cell envelope. The ligand molecules may be parts of the PG itself or other cell wall-associated molecules, thereby significantly impacting the activity range of the enzyme. In some cases, it has been demonstrated that these cell wall binding moieties are situated in the C-terminal part of the endolysin (**Low *et al.*, 2005; Korndörfer *et al.*, 2006; Kikkawa *et al.*, 2007**; etc.). Various conserved binding domains have been described, such as (i) the LysM domain **(Buist *et al.*, 2008; Visweswaran *et al.*, 2011)**, which is to date 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 **(Ohnuma *et al.*, 2008)**; (ii) the bacterial SH3 domain **(Ponting *et al.*, 1999; Whisstock and Lesk, 1999)**; (iii) the choline binding modules of Cpl-1 and other pneumococcal endolysins **(Hermoso *et al.*, 2007)**, which specifically recognize the choline-containing teichoic acid (TA) in the cell wall of *S. pneumoniae*; (iv) the Cpl-1 binding domain, which binds to pneumococcal cell walls in a choline-independent fashion **(Diaz *et al.*, 1991; Bustamante *et al.*, 2010)** and is also present in tandem in the streptococcal λ2A lysin **(Pritchard *et al.*, 2007)**; and the PG-binding domain of two endolysins, Lc-Lys and Lc-Lys2, identified in prophages present in the genome of *Lactobacillus casei* BL23, targeting specifically D-Asn interpeptide bridge of PG **(Regulski *et al.*, 2013)**.
The binding spectrum of a CBD in many cases encompasses an entire bacterial genus and is therefore generally broader than the host range of the respective bacteriophage **(Schmelcher *et al.*, 2012)**. On the other hand, CBD can exhibit specificity down to the serovar or even strain level, as reported for CBDs of *Listeria* phage endolysins **(López and Garcia, 2004; Schmelcher *et al.*, 2010)**. For instance, several different *Listeria* phage endolysins have been characterized and analyzed also for their binding spectra and catalytic mechanism **(Loessner *et al.*, 1995; 1997; 2002; Zimmer *et al.*, 2003; Korndörfer *et al.*, 2006; Schmelcher *et al.*, 2010)** and the individual functional domains can be truncated and recombined to further increase lytic activity and binding affinity **(Schmelcher *et al.*, 2011a,b)**. According to **Loessner *et al.* (2002)**, endolysins exhibit species-specific targeting of the enzyme to its substrate. This targeting is mediated via specific CBDs which recognize certain cell wall components and are thought to be the key to the restricted host range of the enzymes. An example of the different ligand specificity is found among listerial endolysins: in contrast to CBDs from other *Listeria* phage endolysins which directly utilize wall teichoic acid (WTA) as the binding ligand, CBD118, CBD511, and CBDP40 do not require WTA for attachment. Instead, lack of the cell wall polymers enables unrestricted spatial access of CBDs to the cell wall surface, indicating that the abundant WTA can negatively regulate sidewall localization of the CBDs **(Eugster and Loessner, 2013)**.
It has been thought **(Loessner *et al.*, 2002; Briers *et al.*, 2009)** that the interaction between binding domains and ligands is presumably charge-dependent and often characterized by exceptionally high affinity. For CBDs of *Listeria* phage endolysins, equilibrium affinity constants in the pico- and nano-molar range have been determined, which is comparable to or exceeds the affinity of antibodies to their antigens **(López and Garcia, 2004; Schmelcher *et al.*, 2010)**. Thus, endolysins encompass not only a mechanism for killing the cells but also the basis for a specific detection system **(Kretzer *et al.*, 2007; Schmelcher *et al.*, 2010)**, which suggests their possible exploitation.
It was found out that in some cases the absence of the C-terminal domain increased the lytic activity and that the activity could be controlled with the catalytic domain alone **(Mayer *et al.*, 2011)**. A similar pattern was observed for other endolysins where removal of C-terminal CBDs gave more effective endolysins **(Cheng and Fischetti, 2007; Gaeng *et al.*, 2000; Loessner *et al.*, 1998; 1999; Low *et al.*, 2005)**. It seems that the CBD helps mainly the endolysin to direct to the cell wall. Once the endolysin is attached to the cell wall, the CBD hinders the full activity of the catalytic domain. The efficiency of the endolysin depends on the binding kinetics of the CBD. If the binding to the cell wall is strong, then the enzyme can digest only the PG in the immediate surroundings. There are, however, a number of endolysins that show the opposite effect, namely, a decrease in activity when the CBD is removed **(Sanz *et al.*, 1992;**

Loessner *et al.*, 2002; Kikkawa *et al.*, 2007; Porter *et al.*, 2007; Sass and Bierbaum, 2007). In this case, it was proposed that the CBD is needed to help the catalytic domain access the substrate, and the two domains act together to initiate cleavage **(Mayer *et al.*, 2011)**.

Three-dimensional structure and crystal structure of endolysins

While x-ray crystallography is the most commonly used method for protein structure determination, other approaches to determine or predict endolysin structures have been attempted, including NMR techniques **(Kashyap *et al.*, 2012)**, homology modeling **(Sharma *et al.*, 2009; Henry *et al.*, 2011)** or the combination of various biophysical methods **(Bustamante *et al.*, 2010)**. The 3D structures of a handful of single-domain globular phage endolysins have been reported **(Leung *et al.*, 2001; Sagermann and Matthews, 2002; Zhang and Studier, 2004; Xu *et al.*, 2005; Mooers and Matthews, 2006)**. In most cases, only one, mostly the EAD of phage endolysins was determined (**Low *et al.*, 2005; Porter *et al.*, 2007; Sass and Bierbaum, 2007; Korndörfer *et al.*, 2008; Silva-Martin *et al.*, 2010; Mayer *et al.*, 2011)**. Typically, the individual domains of modular endolysins are connected by short and mostly flexible linker regions - this inherent flexibility makes it difficult for complete proteins to crystallize. It is apparent in both Cpl-1**(Hermoso *et al.*, 2003)** and PlyPSA **(Korndörfer *et al.*, 2006)**, the two complete modular endolysins successfully crystallized to date. The PlyC lysin is by far the most active endolysin described to date, yet it is larger than most endolysins and remains the only known endolysin composed of distinct subunits. Although the potent lytic activity of PlyC has been extensively characterized, its structural architecture and mechanism of action has remained unclear **(McGowan *et al.*, 2012)**.

MOLECULAR ENGINEERING OF ENDOLYSINS

Endolysins have been optimized through evolution to cause fast and efficient lysis of the host cell from within, thereby ensuring the phage virion liberation and survival. However, this does not inhibit their potential for improvement applied from without, especially in the complex environment. Gene and protein engineering strategies can alter binding and lytic properties of endolysins and thereby potentially optimize these proteins for specific applications. Table 1 summarizes various types of modifications and their effects on protein properties **(Schmelcher *et al.*, 2012)**.

Table 1 Molecular engineering and its effects on endolysin properties **(Schmelcher *et al.*, 2012)**

Type of modification	*Effect*	*Examples*	*References*
Exchange of CBD	Switch of regulatory properties	LCA and CLL	**Diaz *et al.* (1990)**
	Swapped cell wall specificity	LC7 and CL7 CLC LCL TsI EAD118_CBDPSA EADPSA_CBD118	**Diaz *et al.* (1991)** **Croux *et al.* (1993a)** **Croux *et al.* (1993b)** **Sheehan *et al.* (1996)** **Schmelcher *et al.* (2011a,b)**
	Increased lytic activity	LCL EAD118_CBDPSA	**Croux *et al.* (1993b)** **Schmelcher *et al.* (2011b)**
Fusion of two full-length enzymes	Extended lytic spectrum and increased activity	B30-443-Lyso	**Donovan *et al.* (2006)**
Addition of a heterologous EAD to a full-length enzyme	Extended lytic spectrum and increased activity	B30-182-Lyso	
Addition of a heterologous CBD to a truncated enzyme	Extended lytic spectrum and increased activity	λSA2-E-Lyso-SH3b λSA2-E-LysK-SH3b	**Becker *et al.* (2009a,b)**
Combination of two heterologous CBDs	Extended binding spectrum	GFP_CBD500-P35 GFP_CBDP35-500	**Schmelcher *et al.* (2011a,b)**
Duplication of a CBD	Increased cell wall affinity	GFP_CBD500-500	
	Enhanced lytic activity at high ionic strength	EAD_CBD500-500	
Fusion of an endolysin to a phage minor coat protein	Enhanced solubility	P16-17	**Manoharadas *et al.* (2009)**
Random mutagenesis	Increased lytic activity And shifted salt optimum	PlyGBS90-1	**Cheng and Fischetti (2007)**
Site-directed mutagenesis	Changes in CBD dependence due to altered net charge	PlyBa04-4DFULL PlyBa04-4DCAT	**Low *et al.* (2011)**
	Altered species specificity	L98WCD27L L98WCD27L1-179	**Mayer *et al.* (2011)**

Legend: CBD = Cell wall binding domain; EAD = Enzymatically active domain

DOMAIN RECOMBINATION

The modular architecture of bacteriophage endolysins and the knowledge that was gained from available crystal structures and bioinformatics make it possible to design and create functional protein chimeras consisting of multiple modules of unique origin **(Schmelcher *et al.*, 2010)**. It is undoubtedly true that modular recombination of proteins is one of the driving forces in evolution and permits a rapid adaptation to new environmental conditions. There is an evidence that each domain within such heterologous fusion constructs retains its parental function **(Donovan *et al.*, 2006)**, suggesting that **rational design** and **modular engineering** enable the creation of novel tools with predicted and desired properties for detection and control of pathogens. Particularly, there also exist seemingly natural chimeras such as the *Listeria* phage endolysin PlyPSA **(Zimmer *et al.*, 2003)** or the pneumococcal phage Dp-1 endolysin **Pal (Sheehan *et al.*, 1997)**, indicating that horizontal gene transfer by recombination-driven interchange of endolysin modules occurs naturally within bacteriophage populations or between bacteriophage and bacterial hosts **(Hendrix, 2002)**. For instance, 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 confirm the idea of creating tailor-made antimicrobials and detection tools by harnessing these basic evolutionary principles **(Schmelcher *et al.*, 2012)**.

The groundwork for modular engineering of endolysins was laid in the early 1990s, 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 **(Diaz *et al.*, 1990)**. This meant the earliest approaches to altering properties of PGHs by **domain shuffling**. One of many current approaches to design a chimerical endolysin by domain recombination is that where the advantage of the high solubility of two recently characterized enterococcal endolysins was taken to construct chimeras targeting *S. aureus* **(Fernandez *et al.*, 2012)**. The putative CBD of these endolysins was substitued by a staphylococcal endolysin that showed poor solubility. It was proved that under appropriate conditions the resulting chimeras presented the high solubility of the parental enterococcal endolysins, as well as the broad activity against a collection of the most relevant MRSA epidemic clones and against other Gram-positive pathogens. Some other results of intergeneric fusions were shown by combination of an EAD from the lactococcal phage Tuc2009 with the ChBD of LytA **(Sheehan *et al.*, 1996)**, and more recently by fusion of full-length and truncated versions of the *S. agalactiae* phage B30 endolysin to mature lysostaphin, yielding enzymes that were active against both streptococcal and staphylococcal cells **(Donovan *et al.*, 2006)**.

Fusion of endolysin functional domains to fluorescent marker proteins

Fluorescent proteins (FP) are very popular tools in molecular biology, medicine and cell biology, based on their wide compatibility, lack of toxicity, incredible stability, and no requirement of any cofactors other than oxygen for chromophore formation (**Schmelcher *et al.*, 2010**). Several FP derivatives with shifted spectral characteristics such as blue, cyan, and yellow FP have been developed (**Tsien, 1998; Shaner *et al.*, 2007**). In addition to the GFP variants, several red fluorescent proteins have been described (**Matz *et al.*, 1999; Gurskaya *et al.*, 2001**), e.g. the RedStar protein (RS) (**Knop *et al.*, 2002**). Fusion of a CBD of bacteriophage endolysin with a GFP resulted in heterologous FP fusion constructs that are able to rapidly recognize and brightly decorate a broad spectrum of host cells from species or even serovars (**Schmelcher *et al.*, 2010**). Such chimerical protein constructs offer several advantages, like permitting the visualization of the coating efficiency mediated by GFP reporter gene or replacing the deleted EAD by the GFP and therefore possible use as a required spacer (**Korndörfer *et al.*, 2006**). Subsequently, the detection of cells recognized by the fusion proteins by fluorescent microscopy is easy and elegant to perform (**Loessner *et al.*, 2002; Schmelcher *et al.*, 2010; 2011a,b; Gu *et al.*, 2011**), as seen in Fig. 2. This is a highly desirable property when developing methods for detection of bacterial cells, as well as for high binding affinity, which could be achieved by modular engineering (**Kretzer *et al.*, 2007; Schmelcher *et al.*, 2010; 2012**).

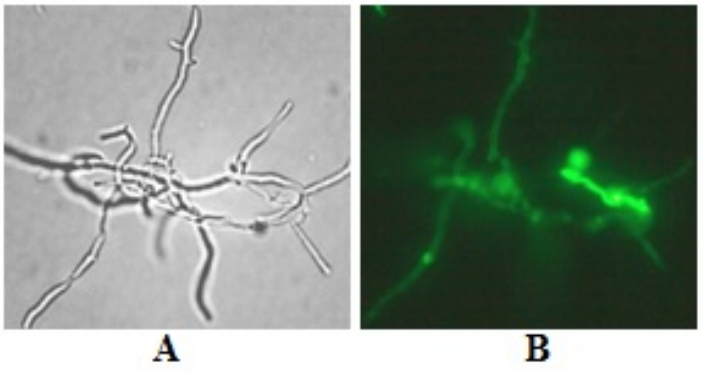

Figure 2 Visualization of the binding activity of the GFP-tagged CBD from Lyt μ1/6 to the cell walls of *S. aureofaciens*.
Fusion protein CBD-GFP was assayed by phase contrast (A) and fluorescence (B) microscopy **(TIŠÁKOVÁ, unpublished)**

RANDOM AND SITE-DIRECTED MUTAGENESIS

In contrast to domain shuffling, where complete functional modules are reassembled and that mimics the naturally occurring horizontal gene transfer among bacteriophages and their hosts, protein evolution can also be directed artificially by inserting point mutations into a parental nucleotide sequence. **Site-directed mutagenesis** is used when the effects of replacing certain amino acids on protein conformation and prediction of protein function are needed to be achieved. **Random mutagenesis** of protein coding sequences, followed by screening of the resulting expression library for clones with desired properties, is another alternative approach (**Schmelcher *et al.*, 2012**).
A basic example for improving the endolysin lytic activity by random mutagenesis was that where the gene encoding 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 (**Cheng and Fischetti, 2007**). In this study, the choice of the screening method 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 could potentially be applied for identification of endolysin mutants with enhanced activity under various conditions (**Schmelcher *et al.*, 2012**).

Another way of improving the enzymatic properties was described in a mutant endolysin CD27L, causing cell lysis of the pathogen *C. difficile*. A point mutation that did not affect the catalytic center was performed. Leu 98 was modified to a Trp residue, which is found in an endolysin from *L. monocytogenes* bacteriohage. This mutation in CD27L endolysin resulted in an increased activity against selected serotypes of *L. monocytogenes*, exhibiting the differences in species specificity compared with the parental protein (**Mayer *et al.*, 2011**).

BIOTECHNOLOGICAL APPLICATIONS OF ENDOLYSINS

Although endolysins are originally designed to work from within infected cells, they work equally well when applied exogenously to Gram-positive cells. Often, a minute amount of purified recombinant enzyme is sufficient to rapidly lyse a dense suspension of cells within minutes or even seconds (**Loessner, 2005**). Owing to unusual substrate specificity and high activity of endolysins, they have been applied to food science, biotechnology and medicine. Endolysins appear to act synergistically when organism-specific enzymes that have different substrate specificities are used in combination (**Loeffler and Fischetti, 2003; Djurkovic *et al.*, 2005; Loessner and Rees, 2005**). Due to the highly specific host lysis and killing, endolysins represent interesting antibacterial agents (**Fischetti, 2005; Loessner, 2005; Borysowski *et al.*, 2006; Hermoso *et al.*, 2007**). Thus, endolysins have been termed protein-antibiotics or "enzybiotics" (**Fischetti, 2005**), and might have a future such (**Loessner, 2005**).
In terms of application there is one regulatory distinction between exploitation of bacteriophages (phage therapy) and endolysins (purified gene products): phages are considered a natural product and endolysins are mostly purified from a recombinant expression system, thus increasing hinders in the approval process. If recombinant enzymes are to fulfill their potential as antibacterials, a number of important factors have to be investigated, such as **drug toxicity**, **immunogenicity**, **efficacy**, **resistance** and **synergy**. To date, a number of both *in vitro* and *in vivo* trials have been carried out on various endolysins to assess these parameters (**Nelson *et al.*, 2012; Pastagia *et al.*, 2013**).

Applications of endolysins in food industry and agriculture

Numerous studies have demonstrated the potential of bacteriophage endolysins for the control and detection of food-borne microbial pathogens (**Borysowski *et al.*, 2006; Callenwaert *et al.*, 2011; Nelson *et al.*, 2012; Schmelcher *et al.*, 2012**). Table 2 compares the possible applications of bacteriocins, bacteriophages and their endolysins in the food industry. The most obvious approach to the use of endolysins for the **biocontrol of pathogens** in food and feed is to directly add purified enzyme to the food or to the raw product as **biopreservatives (Obeso *et al.*, 2008; Garcia *et al.*, 2010)**. For example, the endolysin LysH5 rapidly kills *S. aureus* in pasteurized milk, reducing bacterial numbers below the detection level within 4 h (**Obeso *et al.*, 2008**), and it acts synergistically with the bacteriocin nisin (**Garcia *et al.*, 2010**). Another interesting discovery is the high **thermoresistance** of the three *Listeria* phage endolysins Ply118, Ply511 and PlyP35, which may find applications in food products that undergo heat treatment such as pasteurized milk products (**Schmelcher *et al.*, 2011b**). A less expensive alternative is the production, secretion or release of specific endolysins by starter organisms used in **fermentation** processes, such as *L. lactis* strain (**Gaeng *et al.*, 2000**). In comparison, in the production of cheese, it is hoped that controlled lysis of *Lactococcus* starter strains would result in leaky cells and might aid in accelerated **cheese ripening (Tuler *et al.*, 2002)**.

Endolysins seem to be effective antimicrobials when introduced into foodstuffs via **transgenic expression**, but the safety of consumption of transgenic food products is still a highly debated and assessed topic worldwide (**Maga *et al.*, 2006a,b; Schmelcher *et al.*, 2012**). The generation of transgenic plants that express bacteriophage endolysin genes has been constructed with several agricultural purposes (**Nelson *et al.*, 2012**). The prototype example to achieve resistance to phytopathogenic bacteria *E. carotovora* is the T4 lysozyme potato (**De Vries *et al.*, 1999**).

A very substantial progress has been made in the **detection of food-borne pathogens** using high-affinity CBDs as alternatives to standard detection methods. Thus, endolysins play a relevant role in food safety, based on the high specificity of their CBDs. These recognition domains have been used to develop rapid and sensitive identification, detection, and differentiation systems. It was showed that in foods contaminated with *L. monocytogenes*, detection rates of > 90 % could be reached by CBD-based magnetic separation, using paramagnetic beats coated with recombinant *Listeria* phage CBDs (**Kretzer *et al.*, 2007**).

Table 2 Proposed bacteriocin, bacteriophages and endolysin applications of their antimicrobial activitiy along three main stages of the food chain (based on **García *et al.*, 2010**)

	Production of primary comodities	Food processing	Food storage
Bacteriocins	Prevention/treatment of infections Bacteriocinogenic probiotics Alternative to antibiotic-based growth promoters Reduction of zoonotic bacteria	In situ production of starter/protective cultures or additive/food ingredient Processing aids in cheese ripening and control of fermentation	Extended shelf-life Hurdle technology Active packaging Lower heat treatments Emerging technologies
Bacteriophages	Phage therapy agains infection Reduction of zoonotic bacteria	Fresh-cut produce, infant milk, fermented products Biofilm elimination Food handlers disinfection	Hurdle technology Bacteriocins Devices for pathogen detection

Endolysins	Prophylaxis/therapy Biosanitation of facilities	Preservatives in milk In situ production by starters Biofilm elimination	Devices for pathogen detection and sample enrichment

Applications of endolysins in therapy and biocontrol

The ability of bacteriophages or their products to selectively target pathogenic species of bacteria represents an important advantage compared with antibiotics and the modern medical community is aware of this important fact **(Hermoso *et al.*, 2007; Fischetti, 2010; Nelson *et al.*, 2012; Schmelcher *et al.*, 2012)**. Different types of purified bacteriophage endolysins have been evaluated as potential **anti-infective agents** for the treatment of bacterial infections of humans and animals **(Hermoso *et al.*, 2007; Fischetti, 2010)**. Although it is unlikely that bacteriophages will ever replace antibiotics, they may be useful when no effective antibiotics are available or in conjunction with antibiotics for better treatment of disease **(Schmelcher *et al.*, 2012)**. Since the safe and controlled use of bacteriophages, therapy will require detailed information on the properties and behavior of specific phage-bacterium systems, both *in vitro* and especially *in vivo*.

The first study of the streptococcal phage lysin PlyC to prevent and treat upper respiratory colonization in mice by group A streptococci **(Nelson *et al.*, 2001)**. Another interesting subject, especially with respect to **biowarfare protection**, is endolysin PlyG from the γ-phage of *B. anthracis* **(Schuch *et al.*, 2002)**. There have been also several reports on controlling staphylococcal infections (including MRSA) in animal models within past years **(Rashel *et al.*, 2007)**. Additional enzymes have been described that might be potentially useful for treatment of some infections in humans and animals, such as PlyV12 from an *E. faecalis* phage **(Yoong *et al.*, 2004)** and Ply3626 from *C. perfringens* phage **(Zimmer *et al.*, 2002)**.

The use of endolysins to cause **programmed cell death** and the release of cytoplasmatic content has also several applications, including the production of empty *H. pylori* **(Panthel *et al.*, 2003)** and *E. coli* **(Haidinger *et al.*, 2003)** "ghosts" as vaccine candidates, as well as the programmed self-destruction of intracellular cells of *V. cholerae* or *S. enterica* serovar *Typhimurium* to release antigen-encoding plasmid DNA into the cytosol of eukaryotic cells **(Jain and Mekalanos, 2000)**. Furthermore, an improved version of a **suicide system** for delivery of foreign DNA was presented, based upon conditional synthesis of a *L. monocytogenes* phage endolysin for the destruction of bacterial cells from within infected mammalian cells **(Pilgrim *et al.*, 2003)**.

CONCLUSION

Tightly specific binding and rapid lytic activity places bacteriophage endolysins in the category of peptidoglycan hydrolases effective in bacterial cell wall lysis "from without", with the additional advantage of leaving normal commensal bacteria untouched. Using recombinant DNA technology and molecular engineering approaches, designer protein chimeras could be prepared as a potent source of recombinant endolysins with enhanced and optimalized binding and lytic properties for various applications. In addition, a combination of endolysin anti-bacterial characteristics, as well as the unusual substrate specificity and the high lytic activity, they have been applied to food science, agriculture, biotechnology, biocontrol, therapy etc.

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