Research review paper

Synthetic biology of modular endolysins

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ABSTRACT

Endolysins and their derivatives have emerged in recent years as a novel class of antibacterials, which have now entered the clinical phases. Their rapid mode-of-action and proteinaceous nature differentiates them from any other class of antibiotics. A key feature of endolysins is their modularity and the opportunities that emerge thereof to customize properties such as specificity, activity, stability and solubility. Extensive protein engineering efforts have expanded the activity spectrum to (pan)drug-resistant Gram-negative bacteria or have improved the activity against Gram-positive pathogens. In addition, specific cell wall binding domains derived from endolysins are exploited for the development of diagnostics.

1. Introduction

Endolysins are bacteriophage-encoded enzymes that rapidly degrade peptidoglycan (PG) at the end of the lytic cycle. The infected bacterial cell will lyse and the newly matured bacteriophage particles are dispersed. The natural function of these endolysins can be exploited to use them as powerful enzyme-based antibiotics, also coined 'enzybiotics'. Exogenous application of recombinant endolysin to Gram-positive bacteria rapidly induces osmotic lysis and consequent cell death. In the last two decades, endolysins have proven their efficacy in different models of infection in animals (Nelson et al., 2012; Roach and Donovan, 2015; Schmelcher et al., 2012a) and bacterial contamination in food (Schmelcher and Loessner, 2016). These insights have paved the road towards the (pre)clinical development of endolysins with a few lead endolysins in various phases of preclinical and clinical trials. In a recent comparative analysis with other novel antibacterial approaches, Czaplowski and colleagues ranked endolysins based on their clinical impact and technical feasibility as the alternative class of antibacterials with the greatest potential (Czaplowski et al., 2016). Therefore, the development of endolysins as antibacterials accommodates an important societal need as antibiotic-resistant bacteria are emerging and spreading whilst the antibiotic development pipeline is significantly reduced. This may lead to a post-antibiotic era where we risk losing our ability to treat common infections in both hospital and community environments (WHO, 2016). Indeed, several reports by various global institutes indicate severe human and economic losses if we fail in finding new treatments (Neill, 2014; Shriber et al., 2017; Taylor et al.,

2014).

Possibly one of the most disruptive features of many endolysins is their modular structure and the opportunities that emerge from this modularity to customize endolysins towards their specific application (Fig. 1, summarized in Table S1). Domains of different endolysins have been swapped to modulate specificity or to enhance activity or solubility. Other domains or peptides of diverse origin have been fused to endolysins to provide endolysins with novel functionalities. As a result, applications of endolysins are no longer limited to antibacterials targeting Gram-positive bacteria, but are now expanded to Gram-negative bacteria as well. In addition, applications based on hybrid proteins comprising at least one domain derived from an endolysin are emerging in the field of diagnostics (Schmelcher and Loessner, 2016) and immune-based therapies (Raz et al., 2017). Besides domain swapping, increasing efforts are directed to mutagenesis studies of endolysins. The intimate co-evolution between bacteriophages and bacteria over millions of years has resulted in highly active and specific endolysins. However, there is a discrepancy between their biological function and the function as antibacterial, yielding plenty of margin for protein engineers to perform directed evolution experiments to improve relevant biochemical and biophysical properties.

This review focuses on the recent advances in the synthetic biology of modular endolysins or modules thereof. These synthetic approaches leverage the potential of endolysin-based products to a platform level with a still unknown number of diverse applications targeting any possible bacterium. In addition, we report on the accelerating progress in the (pre)clinical development of endolysins.

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Fig. 1. Synthetic biology of modular endolysins. Typical natural endolysins consist of a single EAD, or have a modular structure comprising at least one EAD and one CBD. Starting from this basis structure, a multitude of variants has been created by protein engineering A. Mutagenesis has been applied and truncations have been constructed to improve activity or to alter specificity. B. Chimeric endolysins comprising CBDs and EADs that are derived from different endolysins have been created with domain swapping. C. Complete endolysins, EADs or CBDs have been fused to several other proteins (fluorescent proteins, receptor binding domains, etc.), peptides or nanoparticles. E.g. Artilysin*s are endolysins fused to a selected peptide. Lysibodies are a fusion between the Fc-fragment of a human antibody and CBDs.

2. The modularity of endolysins

2.1. Structure and function

The structure of endolysins can be either globular or modular. Globular endolysins are unique for phages infecting Gram-negative bacteria, whereas modular endolysins are the rule in phages with a Gram-positive host. Nevertheless, an increasing number of modular endolysins encoded by Gram-negative infecting phages has been identified and characterized, notably often in so-called jumbo phages with a large genome (Briers et al., 2007; Walmagh et al., 2012a). Globular endolysins have a single domain, specifically an enzymatically active domain (EAD) digesting the PG layer. In contrast, modular endolysins are composed of multiple domains with separate activities. Besides one or more EADs, they also comprise a cell wall binding domain (CBD). In some cases, endolysins comprise more than one CBD, e.g. the streptococcal endolysin λ Sa2 has two Cpl-7 CBDs (Pritchard et al., 2007). The majority of modular endolysins found in phages infecting Gram-positive bacteria has the EAD(s) located at the N-terminus and the CBD at the C-terminus, although exceptions are known. E.g., PlySK1249 from a *Streptococcus dysgalactiae* subsp. *equisimilis* SK1249 prophage has a central CBD between two EADs (Oechslein et al., 2013). The different domains are typically linked with flexible interdomain linker(s) of variable length. Modular endolysins in Gram-negative infecting phages have an opposite structure with an N-terminal CBD and a C-terminal EAD (Briers et al., 2009, 2008) or two C-terminal EADs (Oliveira et al., 2013). Comparative genomics of 723 endolysins retrieved from public databases identified 24 different EAD and 13 CBD types arranged in 89 different architectures, underlining the high diversity among endolysins (Oliveira et al., 2013). The CBD of endolysins may have different functions (Schmelcher et al., 2010). Loessner et al. (2002) proposed that the quasi irreversible binding of the CBD to the bacterial cell wall prevents lysis of other possible hosts caused by endolysin diffusion. This hypothesis also explains why the majority of endolysins of phages that infect Gram-negative bacteria are globular. The latter have an outer membrane (OM), which effectively protects bacteria from freely diffusing endolysin molecules after host cell lysis, thus excluding the need

for an irreversibly binding CBD (Briers et al., 2007; Schmelcher et al., 2012a). In addition, a CBD improves targeting of the EAD towards the insoluble PG similar to many other carbohydrate active enzymes. This targeting effect results in a higher enzymatic activity in spite of its irreversible binding and consequently single hit mechanism. This would explain why modular endolysins have on average a five times higher activity than globular ones as shown by the activity analysis of a series of globular and modular endolysins isolated from phages infecting *Pseudomonas aeruginosa* (Walmagh et al., 2012b, 2013).

2.2. Different enzymatic activities

Besides the extensive structural variation, endolysins are also highly diverse in cleavage specificity. They can be divided in five groups (I - V), targeting glycosidic, amide or peptide bonds present in PG. Glycosidases hydrolyze glycosidic bonds and include (I) *N*-acetyl- β -D-glucosaminidases (EC 3.2.1.52), (II) *N*-acetyl- β -D-muramidases (EC 3.2.1.17, also termed lysozymes or muramidases) and (III) lytic transglycosylases (EC 3.2.1.17). The *N*-acetyl- β -D-glucosaminidases target the *N*-acetylglucosaminyl- β -1,4-*N*-acetylmuramine bond at the reducing end of GlcNAc. Both other groups cleave the *N*-acetylmuramoyl- β -1,4-*N*-acetylglucosamine bond, yet, transglycosylases catalyze an intramolecular reaction with the neighboring sugar moieties serving as electron acceptors, resulting in the cleavage of the glycosidic bond between them. No water is involved in this reaction, thus lytic transglycosylases are no hydrolases in contrast to lysozymes. (IV) *N*-acetylmuramoyl-L-alanine amidases (EC 3.5.1.28) hydrolyze the amide bond between MurNAc and the first amino acid of the stem peptide (L-alanine). Finally, (V) endopeptidases (EC 3.4.X.X) can be classified as stem peptide-specific (e.g. L-alanoyl-D-glutamate endopeptidases, γ -D-glutamyl-L-lysine endopeptidases) or interpeptide bridge-specific endopeptidases (e.g. D-alanyl-glycyl endopeptidase). They cut peptide bonds between two amino acids in the stem peptide and the cross-link or -bridge, respectively. Historically endolysin research was focused on endolysins encoded by the *Escherichia coli* T-phages. The first biochemical characterisations were reported for T1, T4 and T7 lysozyme (in addition to λ lysozyme). In addition, T4 lysozyme served as a major

model protein for the study of protein folding. Note that in this period, every endolysin was generally coined a lysozyme without necessarily having the same cleavage specificity as a muramidase according to the contemporary definition of a lysozyme. Since the first description of the use of endolysins as antibacterials against Gram-positive bacteria in vivo studies (Nelson et al., 2001), the focus has been shifted to endolysins active against multidrug-resistant Gram-positive bacteria such as *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Enterococcus faecalis*. Extensive engineering efforts have been done to improve their potential as antibacterial.

3. Engineering of endolysins against Gram-positive bacteria

3.1. Mutagenesis and truncation

Mutagenesis and protein truncation are commonly used to modify the properties of a protein. Both techniques were used to modify the lytic and antibacterial activity, CBD-dependence, specificity, plasma half-life and thermostability of endolysins.

An initially unexpected finding was that the activity of some endolysins can be improved when using a single EAD only. Cheng and Fischetti (2007) used mutagenesis to increase the antibacterial activity of PlyGBS, the endolysin of *Streptococcus* phage NCTC11261, which infects Group B streptococci (GBS). Random mutagenesis of PlyGBS with mutator strains yielded mutants with improved specific activity (18–28-fold) compared to the full-length PlyGBS. These mutants appeared to be truncation mutants, containing an intact N-terminal endopeptidase domain only. The most active mutant, PlyGBS90–1 was tested in an in vivo mouse vaginal model and showed a 4-log reduction over a period of 4 h, compared to a 3-log reduction for wild-type PlyGBS. Also, Horgan et al. (2009) constructed different truncated derivatives of *Staphylococcus* phage endolysin LysK, but with a more rational approach. They found that CHAP_K comprising the cysteine- and histidine-dependent amidohydrolase/peptidase (CHAP) domain, shows a higher lytic activity against staphylococcal cells than LysK in vitro. In addition, CHAP_K was proven to be highly effective in an in vivo mouse nasal model (Fenton et al., 2010). Finally, Low et al. (2011) also demonstrated that the enzymatic activity of some endolysins can be increased upon removal of their CBD. All these observations were in contrast to previous findings, which showed that deletion of the CBD results in inactive variants due to the lack of affinity for the cell wall (Porter et al., 2007). Low et al. (2011) provided an explanation for these contrasting findings and demonstrated that CBD-dependence of a catalytic domain is related to its charge. Using a structure-based engineering approach, they showed that a switch from a negative net charge of the catalytic domain to a positive one, results in an increased interaction with the negatively charged (lipo)teichoic acids in the cell wall of Gram-positive bacteria, and consequently CBD-independence. However, this higher activity is at the expense of specificity since a broader lytic spectrum is obtained in the absence of the CBD. The positive influence of a positive net charge on lytic activity has also been shown in a different way. Díez-Martínez and colleagues substituted 15 amino acids in the cell wall binding-module of Cpl-7, the endolysin of pneumococcal phage Cp-7, thereby increasing the net charge and the lytic activity. The optimized synthetic endolysin is referred to as Cpl-7S (Díez-Martínez et al., 2013).

Mayer and colleagues also truncated CD27L, a *Clostridium difficile* phage endolysin, thereby maintaining only the N-terminal N-acetylmuramoyl-L-alanine amidase domain. The truncation mutant has increased lytic activity against several species that were already sensitive for the full-length endolysin (*C. difficile*, *Bacillus* sp., *Listeria monocytogenes*). In addition, they could successfully further increase the lytic activity by mutagenesis. The leucine residue at position 98 is located between a cluster of three conserved residues that form a hydrogen network at the backside of the catalytic site of the amidase domain. In the homologous endolysin PlyPSA from *Listeria* phage PSA, the

corresponding residue is tryptophan. A single point mutation (L98 W) results in the increased lytic activity against several *L. monocytogenes* serovars, while retaining activity against the other species in the lytic spectrum (Mayer et al., 2011).

Proteins naturally have a short half-life when applied systemically. Resch et al. therefore aimed to increase the plasma half-life. They constructed a dimer of Cpl-1, the endolysin of pneumococcal phage Cp-1, by introduction of a cysteine residue resulting in an intermolecular disulphide bond. The dimer shows an increased lytic activity against *Streptococcus pneumoniae* cells and a reduced plasma clearance in mice, most likely due to the increased molecular weight and thus decreased renal clearance of the dimer (Resch et al., 2011a).

Thermostability is an important property for any protein that is used in medical or industrial applications, as it is directly correlated with the shelf-life. The thermostability of PlyC, the endolysin from streptococcal phage C₁ was improved using different approaches. PlyC has an unusual multimeric structure comprising a central subunit (PlyCA) with two EADs, surrounded by eight identical CBD subunits (PlyCB) that form a ring. The former is responsible for the thermostability of the endolysin (Heselpoth et al., 2015). Therefore, PlyCA was targeted with two approaches to increase the thermostability: directed evolution (random mutagenesis) and computational modeling of the protein stability (site-directed mutagenesis). The former approach resulted in a mutant with a 2.2-fold increase in kinetic stability at 45 °C (Heselpoth and Nelson, 2012), while the latter resulted in a 16-fold increase (Heselpoth et al., 2015). Examples of rationally designed mutant endolysins are limited as this requires knowledge on the tertiary structure of the endolysin, which is only available for a few (but increasing number of) endolysins.

3.2. Synthetic domain recombination or domain swapping

A more disruptive way to alter the properties of endolysins relies on the modular structure of endolysins. The synthetic recombination of endolysin domains from a different origin can lead to chimeric endolysins with altered properties. This approach finds support in the discovery of, what are believed to be, natural chimeric endolysins. Endolysin PlyPSA of *Listeria* phage PSA contains a CBD similar to those of other *Listeria* infecting (pro)phage endolysins. However, its EAD shows no similarity to EADs found in other *Listeria* phages, but rather to EADs found in *Bacillus* and *Clostridium* phages (Zimmer et al., 2003). The endolysin of pneumococcal phage Dp-1, Pal, has a CBD similar to those of other pneumococci infecting phages. Its EAD, however, shows similarity to that of a *Lactococcus lactis* infecting phage, BK5-T (Sheehan et al., 1997). Their formation is made possible by the natural recombination of endolysin domains in the evolutionary process of horizontal gene transfer (Hendrix et al., 1999).

3.2.1. Pioneering research of chimeric endolysins

The proof-of-concept for synthetic domain recombination in endolysins has already been delivered in 1990 by the García group. They demonstrated that the EADs and CBDs of *S. pneumoniae* autolysin LytA and pneumococcal phage endolysin Cpl-1 are interchangeable, resulting in active chimeric enzymes. This implies that the function of each domain is retained upon swapping, and thus that EADs and CBDs can be considered as modules with an autonomous folding and function (Díaz et al., 1990). By swapping the CBDs of LytA and pneumococcal phage endolysin Cpl-7, having respectively a choline-binding and a choline-independent CBD, the cell wall specificity was also swapped (Díaz et al., 1991). CBD swapping is not restricted to bacteria of the same species or genus. This was first demonstrated by Croux et al. by swapping the CBDs of *Clostridium acetobutylicum* autolysin Lyc and pneumococcal phage endolysin Cpl-1 with correspondingly altered specificity (Croux et al., 1993). Another example of an inter-generic recombination is the fusion between the CBD of pneumococcal autolysin LytA and the EAD of the lactococcal phage Tuc2009 endolysin (Sheehan et al., 1996). These examples demonstrate that the domains

used to construct chimeras can also be derived from peptidoglycan hydrolases other than endolysins, such as autolysins (encoded by the bacteria themselves to remodel their peptidoglycan (Johnson et al., 2013)), bacteriocins (produced by bacteria as antibacterial compound against other bacteria (Bastos et al., 2010)) and virion-associated peptidoglycan hydrolases (VAPGHs; structural proteins of the virion that locally degrade the peptidoglycan to allow infection of the bacterial host (Rodríguez-Rubio et al., 2013)). Since this pioneering work, domain swapping has become a common approach to produce customized endolysins. The lytic spectrum of several endolysins has been extended by either CBD addition or swapping. CBDs of endolysins encoded by *L. monocytogenes* phages are often (but not exclusively) featured by a very narrow specificity at the serovar level. Swapping these CBDs (e.g. the CBDs of PlyPSA and Ply118) leads to the corresponding changes in serovar specificity. In addition, combining two such CBDs (e.g. the CBDs from Ply500 and PlyP35) in tandem results in the combined binding spectrum. The binding affinity of a tandem repeat of the same CBD (e.g. the CBD of Ply500) is demonstrated to be 50 times higher than the single CBD (Schmelcher et al., 2011).

3.2.2. Chimeras with improved activity against staphylococci

S. aureus is a major target in the development of chimeric endolysins against Gram-positive bacteria. Domains of LysK, Ply187 and lysostaphin are most commonly used for the construction of chimeras, in addition to several other endolysins. LysK is the endolysin of staphylococcal phage K and is composed of two EADs and a CBD: a CHAP (cysteine, histidine-dependent amidohydrolases/peptidase) domain (N-terminal), an N-acetyl-muramoyl-L-alanine amidase (central) and a SH3b (scr Homology-3) domain (C-terminal). Ply187 comprises an N-terminal CHAP domain and a C-terminal glucosaminidase domain. Lysostaphin is a bacteriocin, encoded by *Staphylococcus simulans* with antibacterial activity against *S. aureus*, harboring an N-terminal glycylglycine M23 endopeptidase domain (EAD) and a C-terminal SH3b domain (CBD). It is encoded by *Staphylococcus simulans* and has antibacterial activity against *S. aureus* (Bastos et al., 2010).

Mao et al. (2013) obtained an increased antibacterial activity against *S. aureus* with a chimeric protein that combines the CHAP domain of Ply187 and the SH3b CBD of LysK. This chimeric endolysin (Ply187AN-KSH3b) has a higher antibacterial activity than full length Ply187, C-terminal truncated Ply187 (CHAP domain) and LysK. All nine-teen staphylococcal strains tested, including methicillin-resistant *S. aureus* (MRSA) strains, are susceptible to the chimera. Ply187AN-KSH3b is also able to disrupt biofilms of *S. aureus*. It was tested in an in vivo *S. aureus* endophthalmitis mouse model. A single intravitreal injection of Ply187AN-KSH3b was able to protect mouse eyes against staphylococcal induced endophthalmitis (Singh et al., 2014). Yang et al. (2014b) made a similar chimeric endolysin, ClyH, by fusing the putative, uncharacterized CBD (not similar to SH3b) of the *S. aureus* phage phiNM3 endolysin (Daniel et al., 2010) to the CHAP domain of Ply187. ClyH exerts lytic activity against all 59 staphylococcal strains tested, among which 56 *S. aureus* strains belonging to both MRSA and MSSA. The in vitro lytic activity of the aforementioned Ply187AN-KSH3b against *S. aureus* is 10-fold higher than that of the Ply187 CHAP domain for the tested strain, while that of ClyH ranges from a 3.7- to 13.6-fold higher activity, depending on the strain. ClyH is able to protect mice from an infection of the peritoneal cavity with *S. aureus* by a single intraperitoneal injection with the chimeric endolysin, which demonstrates its in vivo effectiveness (Yang et al., 2014b). In another study, the same CHAP domain of Ply187 was fused to the C-terminal CBD (SH3-5) of enterococcal phage ϕ 1 endolysin PlyV12, resulting in a chimeric endolysin (Ply187N-V12C) with increased lytic spectrum. PlyV12 exerts lytic activity on multiple *Enterococcus faecalis* and *Enterococcus faecium* strains as well as streptococcal and staphylococcal strains (Yoong et al., 2004). The activity of Ply187N-V12C against staphylococci is similar to that of the CHAP domain of Ply187, but the chimera is also active against some streptococcal and enterococcal

species (Dong et al., 2015). Becker et al. improved the staphylolytic activity of streptococcal λ Sa2 prophage endolysin (λ Sa2lys), which is composed of an N-terminal γ -D-glutamyl-L-lysine endopeptidase domain, two central Cpl-7 CBDs and a C-terminal glucosaminidase domain. By fusing the CBD (SH3b) of either LysK or Lysostaphin to the C-terminus of the λ Sa2lys endopeptidase domain a 5-fold increased activity against *S. aureus* was obtained. Surprisingly, the chimeras also retained activity against streptococci (Becker et al., 2009).

Becker et al. (2016) developed so-called triple-acting lytic peptidoglycan hydrolases. They constructed two triple-acting chimeric fusions by combining domains of LysK and lysostaphin: K-L, where the two EADs of LysK were sequentially fused to the N-terminus of lysostaphin, and L-K, which was constructed by placing the two LysK EADs in-between lysostaphin's endopeptidase and CBD. These chimeric proteins elicit reduced resistance development, which is best explained by the difficulties that an organism experiences to develop resistance against three different activities, each acting on a different bond in the PG structure (see Section 5.1). The in vitro bactericidal activity of these triple-acting constructs is comparable to that of the parental enzymes, both against planktonic cells and biofilms. One of the two tested chimeras, L-K, also showed a significant reduction of *S. aureus* in a rat nasal colonization model.

Finally, Zhou et al. (2017) demonstrated that the deletion mutant LysK Δ 221-390 has a broad lytic spectrum against staphylococci. The mutant is the fusion product of the first 220 (CHAP domain) and the last 105 amino acids (SH3b domain) of LysK. The part that is removed includes the central amidase domain. In a turbidity reduction assay, LysK Δ 221-390 showed lytic activity against all 87 staphylococcal strains tested, including *S. aureus*, *S. hominis* ssp. *hominis*, *S. epidermidis*, *S. haemolyticus*.

A few studies reported on the use of domains of VAPGHs of staphylococcal phages to create chimeric peptidoglycan hydrolases. Rodríguez-Rubio et al. (2013) constructed a chimeric peptidoglycan hydrolase that is highly effective in milk against *S. aureus*. The chimera, named CHAPSH3b, comprises the CHAP domain (EAD) of the VAPGH of *S. aureus* bacteriophage vB_SauS-phiPLA88 (HydH5) and the SH3b domain (CBD) of lysostaphin. The construct is also able to degrade biofilms formed by *S. aureus*. Moreover, subinhibitory doses of CHAPSH3b reduce biofilm formation by *S. aureus* (Fernández et al., 2017). The company Gangagen developed the chimeric enzymatic P128 for intranasal use against *S. aureus*. P128 is a chimeric protein combining the catalytic domain Lys16 of the VAPGH of *Staphylococcus* bacteriophage K with the cell wall binding SH3b domain of lysostaphin (Saravanan et al., 2013; Vipra et al., 2012). P128 will be further discussed in Sections 5.2 and 6.

3.2.3. Chimeras with improved activity against pneumococci

Besides *S. aureus*, *Streptococcus pneumoniae* has been a major target for synthetic endolysins. Díez-Martínez et al. (2015) swapped the modules (EAD, CBD and linker) of two pneumococcal phage endolysins: the synthetic endolysin Cpl-7S (see Section 3.1) and the natural endolysin Cpl-1. The chimeric variant Cpl-711 composed of the EAD of Cpl-7S and the linker and CBD of Cpl-1 has the highest bactericidal activity of all possible chimeras and the parental endolysins against all six *S. pneumoniae* strains tested, thereby outperforming the most powerful lytic enzyme against pneumococci, i.e. Cpl-1. Cpl-711 shows improved in vitro activity against planktonic pneumococci, compared to Cpl-1, but also in vitro against pneumococcal biofilms and in vivo in a mouse bacteraemia model. Later, the same research group constructed the chimeric endolysin PL3, which compares to or surpasses the lytic activity of Cpl-711 against pneumococci, depending on the strain. PL3 lyses all five pneumococcal strains tested as well as several non-pneumococcal streptococcal strains with a choline-containing cell wall (*S. oralis*, *S. pseudopneumoniae* and *S. mitis*). The two parental enzymes are pneumococcal phage endolysin Pal and the major pneumococcal autolysin LytA. The architecture of both peptidoglycan hydrolases is the

same, i.e. an N-terminal EAD, a linker and a C-terminal CBD. Their CBDs both comprise six choline-binding repeats and a C-terminal tail. PL3 is composed of the EAD, linker and first two choline-binding repeats of Pal, while the latter four choline-binding repeats and the CBD tail originate from LytA. The junction in this new chimeric protein is thus located within an existing domain. This is possible because of the unique repetitive structure of this CBD. The choice to include the linker and first two repeats of Pal was made to preserve the Pal overall modular structure. The *in vivo* efficacy of PL3 has been successfully demonstrated in a zebrafish embryo infection model (Blázquez et al., 2016).

3.2.4. Increasing the solubility

Several endolysins suffer from a low solubility during or after expression. Manoharadas et al. (2009) encountered this upon recombinant production of *S. aureus* phage P68 endolysin P16. To overcome the low solubility, the D-alanyl-glycyl endopeptidase domain of P16 was fused to the C-terminal CBD of the VAPGH P17 of the same phage. The resulting P16–17 chimera is now soluble and shows muralytic and anti-bacterial activity towards *S. aureus*. Similarly, the endolysin of *S. aureus* phage F87s/06 Lys87 precipitates during expression due to low solubility. To improve the low solubility of Lys87, Fernandes et al. (2012) made use of the high solubility of the endolysins of *E. faecalis* phages F170/08 and F168/08: Lys170 and Lys168. They replaced the CBD of Lys87 with either the CBD of Lys170 or Lys168, resulting in the respective chimeric endolysins Lys168–87 and Lys170–87 with increased solubility. The chimeras lyse all strains of a representative set of 43 typed *S. aureus* clinical isolate strains. Also, they can lyse several *E. faecalis* and *E. faecium* clinical isolates similar to the parental endolysins Lys170 and Lys168. Additionally, they can lyse several staphylococci (other than *S. aureus*) and streptococci. Later on, the solubility of recombinantly expressed wildtype endolysins acting against *S. aureus* (e.g., P68, LysK) could be improved by modifying the expression and purification protocols, a.o. by including 30% glycerol in lysis, wash and elution buffers (Schmelcher et al., 2015b).

3.2.5. Increased activity under physiological conditions

An important property to consider when testing (chimeric) peptidoglycan hydrolases is the ionic conductivity of the buffer. The EAD of *S. aureus* autolysin LytM has an optimal activity under low conductivity (< 3 mS/cm) media, which poses a problem for the use as therapeutic under physiological conditions. Mature lysostaphin on the other hand, only performs well under high conductivity (> 10 mS/cm). (Jagielska et al., 2016) fused LytM to the CBD of lysostaphin (SH3b), either N- or C-terminally. Both chimeras have a higher activity under high conductivity than LytM but similar to lysostaphin. The lysostaphin CBD thus transfers the capacity to perform better under high conductivity. The authors hypothesize that the increased activity due to the addition of the CBD is due to enhanced peptidoglycan binding under higher ionic conditions.

3.2.6. High-throughput analysis of chimeric or truncated endolysins

A few studies analyzed in a more systematic approach the activity of broad set of chimeric variants. Verbree et al. (2017a) screened a library of > 170 endolysins and other peptidoglycan hydrolases, both natural and chimeric, against *S. aureus* in milk in a single study, allowing comparison on a large scale. Nine promising candidates were identified in a high-throughput microtiter plate-based screening assay, which were further compared in time-kill assays. Two constructs fully eradicate *S. aureus* at a low enzyme concentration (360 nM): lysostaphin and CHAPK_CWT-LST. The latter is a chimera comprised of the CHAP domain of LysK at the N-terminus and the SH3b CBD of lysostaphin at the C-terminus (Verbree et al., 2017a, 2017b).

Yang et al. (2015a) developed a novel screening method based on induced-lysis for the rapid expression and screening of a large number of endolysins. They constructed a library of random chimeric

endolysins, starting from 7 EADs and 3 CBDs originating from endolysins with different specificities (*S. aureus*, streptococci, *Listeria*, enterococci and *Acinetobacter baumannii*). Each construct contained one of the seven EADs at its N-terminus and one of the three EADs at its C-terminus. Basically, the *E. coli* cells used in the assay contains two plasmids, one encoding the ClyN gene and one with the chimeric endolysin gene, both under control of a different promoter. First, the chimeric endolysin is expressed by induction with arabinose (pBAD promoter). Then ClyN is expressed by induction with IPTG (T7 promoter). ClyN will lyse *E. coli* from within upon expression, resulting in the release of the chimeric endolysin. The lysate can then be directly used for an on-plate bactericidal screening assay. Using this method, a new chimeric endolysin, ClyR, with a broad lytic spectrum, primarily against streptococci, was identified. The N-terminal part of ClyR is the CHAP domain (EAD) of the PlyCA subunit of PlyC. Its C-terminus is the SH3b domain (CBD) of a *Streptococcus suis* prophage endolysin, PlySs2 (also referred to as CF-301). Further characterization demonstrated that ClyR lyses multiple streptococcal strains (*S. pyogenes*, *S. agalactiae*, *S. dysgalactiae*, *S. equi*, *S. uberis*, *S. mutans*, *S. pneumoniae*, *S. suis*, *S. cristae*, *S. gordonii*, *S. intermedius* and *S. parasanguinis* strains), but also *E. faecalis* and *S. aureus*. The *in vivo* efficacy of ClyR was demonstrated in a mouse systemic *S. agalactiae* infection model (Yang et al., 2015a), similar to the one described above for ClyH. ClyR is capable to reduce the number of *S. mutans* in biofilms (in vitro) with 2–3 log units, depending on the conditions. This activity translates to a significant reduction of dental plaques in an *in vivo* mouse dental colonization model with *S. mutans* (Yang et al., 2016). Using the same method and library, a chimeric endolysin (ClyF) was identified with robust lytic activity against MRSA, both planktonic cells and in biofilms (in vitro). The chimera consists of the N-terminal CHAP domain of Ply187 and the SH3b domain of PlySs2. ClyF has an increased thermostability and pH tolerance, compared to the CHAP domain of Ply187. In PBS buffer, the antibacterial activity of ClyF against MRSA is comparable to that of the CHAP domain of Ply187. However, in bio-matrices (in serum, milk and on pigskin) the antibacterial activity of ClyF is remarkably higher. *In vivo* efficacy of ClyF has been demonstrated for planktonic MRSA in a mouse systemic infection model (similar to the model used for ClyH and ClyR). *In vivo* biofilm degradation activity of ClyF against MRSA has been demonstrated in a mouse model of burn wound infection (Yang et al., 2017).

3.3. Cell wall binding domains used for detection

Antibodies can be successfully used to detect (pathogenic) bacteria by specific binding to one or more epitopes (Singh et al., 2011). Nevertheless, they may suffer from drawbacks such as a possible lack of specificity and sensitivity and they are prone to activity loss in different matrixes. Moreover, their production is laborious, expensive and time-consuming, as it involves the immunization of animals and maintenance of hybridoma cells (Singh et al., 2012; Skottrup et al., 2008). CBDs provide a valuable alternative for detection of bacteria. They target a specific cell wall epitope with high affinity (pico- to nano-molar range) and their binding sites are highly abundant on the bacterial surface (at least 10^7) (Schmelcher et al., 2012a; Yang et al., 2012). CBDs (10–20 kDa) are generally smaller than antibodies (150 kDa) and can be easily expressed and purified in prokaryotic expression systems. Endolysin CBDs are thus an inexpensive, host-specific alternative for antibodies (Kong and Ryu, 2015; Yu et al., 2016). Notably, they can only be applied against Gram-positive bacteria, as the OM shields the PG layer, which is the target of CBDs of modular endolysins of Gram-negative infecting phages (Bai et al., 2016; Schmelcher and Loessner, 2016).

3.3.1. Detecting and separating bacterial cells

Detection of foodborne pathogens has a pivotal role in food industry. The most important Gram-positive food pathogens are *Clostridium perfringens*, *L. monocytogenes* and *S. aureus*. Together with

some Gram-negative species (e.g. *Salmonella*, *Escherichia coli*, etc.), they cause severe economic losses, foodborne illness and death (Bai et al., 2016; Schmelcher and Loessner, 2016). Therefore, the food industry needs sensitive and specific diagnostic tools to reliably and rapidly identify microbial contamination. Moreover, the methods need to be inexpensive from an economical point of view and easy to use (Kretzer et al., 2007). Current methods are primarily based on culturing on specific media, PCR- or antibody-based detection, but are often time-consuming and labor-intensive (Hagens and Loessner, 2007). An important disadvantage of PCR-based methods, is their inability to differentiate between live and dead cells. This is essential for diagnostics in food, as a PCR would produce positive signals even when pathogens have been inactivated, e.g. by heat treatment. In addition, PCR-based detection may be inhibited by the complex (food) matrix. Kretzer et al. exploited the properties of CBDs to specifically separate *Listeria* from contaminated food samples. Specifically, the CBDs from *L. monocytogenes* phage endolysins Ply118 and Ply500, which both recognize all serovars, were fused to magnetic beads (CBD-MS) to separate *Listeria* cells from the contaminated food. This detection method is superior to conventional detection techniques in terms of sensitivity (0.1–100 CFU/mL) and required time (< 48 h vs. 96 h). Moreover, the CBD-MS specifically capture > 90% of the bacteria in the presence of heterogeneous bacterial suspensions and are functional in various food samples including turkey breast, ground meat, salmon, cheese, iceberg lettuce and milk (Kretzer et al., 2007). The CBD-MS method was integrated with real-time PCR detection and quantification, specifically for the detection of *L. monocytogenes* from milk (Walcher et al., 2010). An alternative approach to differentiate *Listeria* serovars is based on the variable binding spectra of serovar-specific CBDs. A toolbox of fusion proteins of different CBDs and fluorescent proteins was created that allows multiplex staining of *Listeria* cells in mixed cultures. Thereafter, the cells can be visualized and differentiated by fluorescence microscopy. Moreover, blending the multiplex staining and CBD-MS approach enables simultaneous capturing and differentiation of multiple *Listeria* serovars from contaminated milk and camembert cheese samples in < 15 min (Schmelcher et al., 2010). The CBD-MS approach has also been successfully extended to other Gram-positive species such as *B. cereus* with CBD21 and *C. perfringens* with CBD3626 (Kretzer et al., 2007).

Yu and colleagues developed a CBD-based magnetic enrichment immunoassay to detect *S. aureus*. Immunomagnetic particles (IMPs) were coated with IgG antibodies to capture *S. aureus* through staphylococcal protein A binding. As such the bacteria were concentrated and possible matrix interference during detection was excluded. The biotinylated fusion protein T-CBD of the red fluorescent protein tdTomato and the specific *S. aureus* CBD, PlyV12C, served as the second ‘antibody’ in the set-up. Finally, the streptavidin linked horseradish peroxidase was used to further increase the sensitivity of detection. This setup resulted in a detection limit in contaminated milk of 4×10^3 CFU/mL in 1.5 h (Yu et al., 2016).

Another group developed an electrochemical impedance sensor through immobilization of the CBD of *L. monocytogenes* phage endolysin Ply500 onto a gold sensor surface. Impedimetric detection of *Listeria* cells by electrochemical impedance spectroscopy is fast (± 20 min), both in buffer and artificially contaminated milk, with a detection limit of 10^4 CFU/mL and 10^5 CFU/mL, respectively (Tolba et al., 2012). Kong et al. integrated a CBD specific for *B. cereus* (LysPBC1) on a surface plasmon resonance (SPR)-chip and this resulted, by using a subtractive inhibition assay, in a detection limit of 10^2 CFU/mL (Kong et al., 2015). A major drawback of both methods is the requirement for sophisticated, expensive equipment (Schmelcher and Loessner, 2016). This was avoided through the development of a nitrocellulose based lateral flow assay (LFA) for the detection of *Bacillus cereus* using the CBD of endolysin LysB4 from *B. cereus* phage B4. Briefly, the LFA works as follows. First, the nitrocellulose membrane is dipped in a solution containing *B. cereus*. The bacteria will bind to the CBDs immobilized at

the test line in the membrane. Visualisation occurs in the second step, when the membrane is dipped in a solution with gold nanoparticles. These nanoparticles are coated with cysteine-glutathione-S-transferase-tagged CBDs (Cys-GST-CBD-AuNP) and will visualize the bacteria by producing a red test line. Summarizing, the authors developed a simple, sensitive (10^4 CFU/mL), rapid (20 min) and cost-effective bisosensor based on CBDs (Kong et al., 2017).

3.3.2. Targeted binding by CBDs

Lui and colleagues used the high affinity and specificity of the serovar-specific CBD of *Listeria* phage endolysin PlyP35 for a targeted delivery of a bacteriocin to *Listeria* cells. They engineered a Leucocin C secreting *E. coli* strain with the chimera FliC::CBDP35, resulting in the formation of flagella that display the CBDP35 domain. This engineered strain effectively binds to *Listeria* cells through its flagella and subsequently kills them by the secreted Leucocin C, resulting in a 2 log reduction (Liu et al., 2013). The authors applied the same principle to pediocin-secreting *E. coli* cells with FliC::CBD500 targeting *Listeria* cells as well. However, the FliC fusion protein hindered the formation of functional flagella (Liu et al., 2015).

In a recent paper, Raz and his colleagues demonstrated how the binding properties of CBDs can be used to enhance the human immune system in killing drug-resistant bacterial pathogens. One of the most abundant components of the Gram-positive bacterial cell wall are carbohydrates from (lipo)teichoic acids. Although these carbohydrates can be recognized by therapeutic antibodies, they are only poor immunogens. Yet, CBDs recognize these targets with high affinity. Therefore, the authors fused the binding domain of an autolysin (R1–R2 binding repeats of the major staphylococcal autolysin AtlA) or a CBD (of endolysin ClyS or PlySs2) to the Fc-fragment of the human antibody IgG1, creating so-called lysibodies. The CBD of the lysibody specifically binds to the bacterial pathogen, followed by recognition of the bound Fc-fragment by the immune system. This results in immune-based opsonization by macrophages or neutrophils. Bound lysibodies also lead to complement deposition on the surface. The ClyS lysibody significantly reduces the number of *S. aureus* bacteria in a kidney abscess model, whereas mice have significantly improved survival rates when they are treated with the AtlA lysibody in a bacteremia model. Moreover, the authors expect a low probability of resistance development against lysibodies as resistance implies that the autolysin will be hampered in performing its natural function in the cell wall metabolism and resistance development against (CBDs of) endolysins is generally considered as low. In addition, lysibodies can be potentially developed towards any Gram-positive bacterium, as the pool of CBDs is almost inexhaustible and can be expanded towards other disease-causing pathogens such as viruses, parasites or fungi (Raz et al., 2017).

4. Endolysins against Gram-negative bacteria

4.1. Intrinsic ability of endolysins to cross the OM

The lack of therapeutic options is most concerning for multidrug-resistant Gram-negative pathogens. Antimicrobial resistance has risen in almost all pathogenic species and the first pandrug-resistant strains have emerged with the appearance of colistin-resistant *E. coli* strains. Moreover, during the last decades, no new antibiotics classes have been approved for Gram-negative infections (Fair and Tor, 2014; Luepke et al., 2017). Gram-negative bacteria are intrinsically resistant to the antibacterial action of endolysins due to the presence of an OM. The OM acts as a semi-permeable membrane (cutoff ~ 600 Da via non-specific porins) that shields access to the PG layer. Nevertheless, different endolysins have been reported that have an intrinsic antibacterial activity against Gram-negative pathogens such as *Acinetobacter baumannii* (LysAB3 and LysAB4 (Lai et al., 2013), PlyAB1 (Huang et al., 2014), PlyF307 (Lood et al., 2015), LysABP-01 (Thummeepak et al., 2016), ABgp46 (Oliveira et al., 2016b), LysPA26 (Guo et al., 2017)),

Pseudomonas aeruginosa (LysPA26 (Guo et al., 2017)), *Citrobacter freundii* (CfP1 lysin (Oliveira et al., 2016a)), *E. coli* (T5 endolysin (Shavrina et al., 2016), SPN9CC (Lim et al., 2014), LysPA26 (Guo et al., 2017)) and *K. pneumoniae* (LysPA26 (Guo et al., 2017)). A comparison with endolysins acting against Gram-positive species shows that for many of these endolysins higher doses (100–500 µg/mL vs. < 10 µg/mL) are required for killing (Lim et al., 2014; Shavrina et al., 2016; Thummeepak et al., 2016) or that killing is less extensive (Oliveira et al., 2016b; Walmagh et al., 2012b). This indicates that endolysin molecules penetrate the OM but are still significantly hampered by the barrier. For a currently unknown reason, *A. baumannii* appears more susceptible for these endolysins than other Gram-negative species. PlyF307 has a potent antibacterial activity (> 5 log reduction) against all tested clinical strains of *A. baumannii*. Exposure to PlyF307 results in a 1.6 and 2 log reduction of CFU in an in vitro and in vivo mouse catheter biofilm model, respectively. Moreover, PlyF307 significantly improves the survival rate of mice in a sepsis model (Lood et al., 2015).

Cationic or amphipathic regions appear to be a common structural element at the C-terminus of these endolysins with intrinsic activity as shown by sequence analyses, truncation or mutagenesis studies (Lai et al., 2013, 2011; Lood et al., 2015; Thandar et al., 2016). A deeper understanding how these endolysins are able to pass the OM could provide insights in how to engineer endolysins in order to tailor their applicability towards Gram-negative pathogens (Lood et al., 2015).

4.2. Peptides derived from endolysins

Recent efforts focus on the use of peptides derived from endolysins as antibacterials, since some appear to have similar physicochemical properties as reported for antimicrobial peptides (AMPs). The mode-of-action is here no longer based on peptidoglycan degradation and osmotic lysis, but on membrane disruption similar to other AMPs. Thandar et al. (2016) identified a highly positively charged C-terminal domain in PlyF307 and showed that this peptide (P307) possesses a potent antibacterial activity against *A. baumannii*, causing a 3 log reduction in cell number. Four peptides of eight amino acids were fused to the C-terminus of P307. P307_{SQ-8C}, a fusion of eight amino acids (SQSRESQC) of the hepatitis B virus core protein to the C-terminus of P307, has the highest antibacterial activity (> 5 log reduction in cell number). The short peptide (SQSRESQC) does not possess bacterial activity on its own, nor it increases the antibacterial activity of P307 if added in equimolar concentrations, showing the benefits of direct fusion. Moreover, scrambling the SQSRESQC amino acid sequence in P307_{SQ-8C} does not affect the antibacterial activity. This demonstrates that the composition of the small peptide is more important than its sequence. The highest bactericidal effect of P307 and P307_{SQ-8C} is seen against *A. baumannii* strains, although also *Bacillus anthracis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were sensitive (~ 1.3 to 2.9 log decrease in cell number). Whereas *E. coli* and *K. pneumoniae* are insensitive at physiological pH, they become sensitive at pH 8.8. The growth phase of *A. baumannii* does not affect the antibacterial activity of P307_{SQ-8C}. P307_{SQ-8C} has also a high activity against *A. baumannii* biofilms. P307 and P307_{SQ-8C} kill bacteria by destabilizing the cytoplasmic membrane (CM), but are not hemolytic. However, the bactericidal activity of both peptides is completely lost when tested in human blood plasma. Yet, P307_{SQ-8C} reduces the bacterial counts by 2 log units in a in vivo mouse skin model, the predominant of infection route of *A. baumannii* (Thandar et al., 2016).

4.3. OM permeabilization by high pressure or chemicals

To facilitate passage of endolysins across the OM, several approaches have been evaluated. Chelating compounds such as EDTA organic acids (Briers et al., 2007; Oliveira et al., 2016b; Yang et al., 2015b), essential oils (Chang et al., 2017; Díez-Martínez et al., 2013), high hydrostatic pressure (Briers et al., 2008) and antibiotics

(combination between colistin and LysABP-01 (Thummeepak et al., 2016)) have been successfully demonstrated to increase the bactericidal effect of endolysins against diverse Gram-negative pathogens. Colistin or polymyxin E, can act as an OM permeabilizer due to its cationic peptide ring, which interferes with the negatively charged phosphates in the lipid A moiety of the lipopolysaccharide and displaces the divalent cations (Mg²⁺ and Ca²⁺). This destabilization of the OM promotes endolysin passage, resulting in a synergistic effect (Thummeepak et al., 2016). Carvacrol, an essential oil of oregano and thyme, is able to disintegrate the OM, thereby creating access for the endolysins to the PG. This has been shown for the engineered pneumococcal lysozyme Cpl-7S that efficiently kills Gram-negative bacteria (*E. coli*, *P. putida*) in the presence of 0.01% carvacrol (Díez-Martínez et al., 2013). Yet, carvacrol also shows synergy with endolysin LysSA97 against *S. aureus* in foods (Chang et al., 2017). These approaches are often not applicable for animal or human therapies but rather aim at food applications. High hydrostatic pressure, for example, is suited for food products that cannot undergo thermal conservation techniques, such as oysters and guacamole. Combination with endolysins reduces the required pressure, and correspondingly the related equipment and energy costs (reviewed in Briers and Lavigne, 2015 and Schmelcher et al., 2012a).

4.4. Expanding the spectrum of endolysins as antibacterials towards Gram-negatives

Protein engineering has been used to expand the antibacterial spectrum of endolysins to Gram-negative bacteria. Two different approaches have been used: (i) fusion with outer membrane permeabilizing peptides (Briers et al., 2014b) and (ii) fusion with a receptor-binding domain (P. Lukacik et al., 2012a, 2012b; Patzer et al., 2012).

4.4.1. Outer membrane permeabilizing peptides

The OM is stabilized by divalent cations (e.g. Mg²⁺ and Ca²⁺) through ionic interactions with the phosphate groups of adjacent LPS molecules and by hydrophobic stacking of the lipid A moiety of LPS molecules. This results in an impermeable barrier for both hydrophobic and hydrophilic compounds larger than 600–650 Da that cannot cross via the non-specific porins (Briers and Lavigne, 2015). Application of a fusion protein of an endolysin with a selected outer membrane permeabilizing (OMP) peptide leads to local destabilization of the OM, followed by passage of the fusion protein across the OM and degradation of the peptidoglycan layer. This results in immediate cell death through osmotic lysis (Briers et al., 2014b). The OMP-peptide can have polycationic, hydrophobic or amphipathic properties, allowing them to interfere with the ionic and hydrophobic stabilizing forces of the OM. These fusion proteins are called ‘Artilysins’ and act independently of protein transport systems (Briers et al., 2014b). Artilysin® is a registered trademark in the European Union, United States and other countries, owned by Lysando AG.

In an initial study, seven (putative) LPS-destabilizing peptides, either polycationic, hydrophobic or amphipathic, were fused to the N-terminal end of two modular endolysins, OBPgp279 and PVP-SE1gp146. LoGT-001, a fusion of a polycationic nonapeptide (PCNP) to OBPgp279, has the highest antibacterial activity against *P. aeruginosa* (2.6 log reduction in 30 min) compared to the original endolysin OBPgp279, which has an intrinsic antibacterial activity (1.1 log reduction in 30 min). The addition of 0.5 mM EDTA has a significant synergistic effect on the antibacterial activity (5.4 log reduction in 30 min). Lysis of *P. aeruginosa* PAO1, visualized using time-lapse microscopy, takes place within minutes. Immediately after addition of the LoGT-001, membrane bulging and cell degradation (i.e., lysis) was observed, thus confirming the postulated mode-of-action, i.e., OM destabilization, translocation to the periplasm and degradation of the PG layer resulting in osmotic lysis. Also, the multidrug-resistant strain Br667 is effectively killed by LoGT-001 in the presence of 0.5 mM EDTA (4.27 log units). In a systematic approach, the effects of the linker

length/composition, endolysin moiety and peptide moiety were analyzed. Both peptide, linker and endolysin significantly affect the antibacterial activity. From this study, a few design rules could be extracted: (i) an increasing linker length is beneficial for the bactericidal activity, presumably due to a concomitantly increased autonomy of the destabilizing peptide and endolysin moieties. (ii) In the case of OBPgp279, N-terminal fusions of the OMP-peptide result in more active antibacterials compared to C-terminal fusions. In the case of Enterobacteriaceae (*E. coli* and *Salmonella* Typhimurium) a tandem fusion of a PCNP and a hydrophobic or amphipathic peptide increased the activity significantly more than fusions with single peptides (Briers et al., 2014b). This study has shown the strength of the synthetic biology of modular Artilyns (OMP-peptide, linker, CBD, EAD) with the potential to engineer the specificity and properties towards any bacterial species and/or condition (Briers and Lavigne, 2015). Furthermore, PCNP-based Artilyns significantly reduce the bacterial load in an in vitro skin infection model using seeded human keratinocytes that are infected with *P. aeruginosa* and they are able to fully protect the skin monolayer without any cytotoxic effect. In addition, *C. elegans* worms infected with the highly virulent *P. aeruginosa* PA14 strain and treated with LoGT-008 show an increased survival rate (63%) compared to the untreated control (10%) and the positive control with ciprofloxacin (45%) (Briers et al., 2014b).

The majority of antibacterial AMPs are amphipathic allowing for self-mediated uptake through the OM of the bacteria. Subsequently the cell is killed by targeting intracellular components or by simple CM disruption. Briers et al., 2014a demonstrated that fusion of SMAP-29, an α -helical and amphipathic AMP originating from sheep myeloid, to the N-terminus of the KZ144 endolysin (Art-085) also results in highly bactericidal enzybiotics (4 log reduction of *P. aeruginosa* in 30 min). Subsequently, three cysteines of KZ144 were mutated to serine to avoid oligomer formation and to increase the structural stability and activity of the Artilyns, resulting in Art-175. Art-175 is effective against all 79 tested *P. aeruginosa* strains, including multidrug-resistant isolates, with a MIC comparable to traditional antibiotics (0.3 μ M). Similar to most endolysins, resistance development could not be provoked upon exposure to sublethal doses in contrast to control antibiotics. In addition, no cross-resistance was observed for 13 prevalent resistance mechanisms, including the recently emerging mcr-1 colistin resistance mechanism. The latter finding is particularly relevant for two reasons: (1) both Art-175 and colistin initially target the ionic interactions stabilizing the outer membrane, which could be a molecular base for cross-resistance; (2) it is important that novel experimental antibacterials are active on those isolates that are at the highest risk to develop pan-drug resistance (Schirmeier et al., 2017). Metabolically quiescent persister cells remain susceptible to the antibacterial activity of Art-175 in contrast to standard first line antibiotics, explaining why Artilyns can eradicate cultures completely in contrast to antibiotics where a remaining subpopulation remains unaffected. If these cells are not killed by the human immune system during an infection (e.g. when they are embedded in a biofilm), they can result in a recurrent, chronic infection. This observation can be best explained by the mode-of-action of Artilyns based on the active disintegration the PG in contrast to standard antibiotics that passively inhibit active and essential metabolic processes (Briers et al., 2014a). No cytotoxicity was observed for Art-175 using L-929 mouse connective tissue fibroblasts. Besides, *P. aeruginosa* Art-175 kills stationary cell of 32 *A. baumannii* strains and one *Acinetobacter lwoffii* strain, including multidrug-resistant ones (up to 9 log reduction in cell number), again underlining the remarkably high susceptibility of *Acinetobacter* species as has been observed for endolysins with intrinsic antibacterial activity (Defraigne et al., 2016). In vivo efficacy of Art-085 was demonstrated in two case studies of dogs with ear infections. Three *P. aeruginosa* strains together with a *Streptococcus* sp. strain were identified in the ears of one dog and two *P. aeruginosa* strains together with a *Proteus mirabilis* strain in the ears of the other dog. Treatment with relevant antibiotics, during respectively

six and three weeks, could not heal the infections. However, treatment with Art-085 was effective without relapse (Briers and Lavigne, 2015).

Other fusions between endolysins and peptides to improve the antibacterial activity against Gram-negative pathogens have been reported. Yang et al. (2015b) constructed the peptide-modified endolysin (PlyA), based on a fusion between the residues 1–8 (KWKLFFKKI) of the polycationic peptide cecropin A and endolysin OBPgp279. PlyA has a high antibacterial activity against *A. baumannii* and *P. aeruginosa* with 5 and 4 log reduction in cell number, respectively. This activity was observed irrespective of any present resistance mechanism, but the growth phase of the bacterium had a major effect on the antibacterial activity in contrast to Art-085. Both EDTA and citric acid enhanced the antibacterial activity against stationary phase *A. baumannii* and *P. aeruginosa* cells. No antibacterial activity was observed in bio-matrix conditions (Yang et al., 2015b). Ma and his colleagues investigated whether fusions of peptides with different length and charge (e.g. cationic and/or hydrophobic amino acids) to the C-terminus of the *E. coli* phage endolysin Lysep3 could enhance the bactericidal activity against *E. coli*. Specifically, Lysep3 was fused to four polycationic peptides of a different length between 5 and 15 amino acids and one hybrid peptide of five cationic and seven hydrophobic amino acids, each resulting in a fusion protein with a different isoelectric point. It was found that the length of the polycationic peptide increases the bactericidal effect, with the activity of the hybrid peptide (12 amino acids) falling in between the effect of the polycationic peptides of 10 and 15 amino acids. The addition of outer membrane permeabilizers (e.g. EDTA and citric acid) further increases the antibacterial activity 1.1–1.5 fold. The best fusion endolysin, Lysep3 with 15 amino acids, has an antibacterial activity (between 8 and 43% reduction) against all tested strains, including twelve *E. coli* isolates and three other Gram-negative bacteria (*Salmonella*, *Shigella*, *P. aeruginosa*) (Ma et al., 2017).

Also, the addition of a domain containing a positively charged C-terminus can enhance the antibacterial activity of endolysins towards Gram-negative species. Orito et al. (2004) showed that the C-terminal CBD (D8) of the *Bacillus amyloliquefaciens* lysin D1 contains two amphiphilic helices and increases the permeability of the OM. A fusion protein of D8 to the N-terminus of Lysep3 has an in vitro bacteriocidal activity against Gram-negative (*E. coli*, *P. aeruginosa* and *A. baumannii*) and Gram-positive species (*Streptococcus*) (Wang et al., 2017).

'Artilynsation' of endolysins has also been successfully applied on a Gram-positive-specific endolysin as an approach to improve its properties. Rodríguez-Rubio et al. (2016) demonstrated that Artilyns-240 (Art-240), a fusion of the polycationic peptide PCNP to the C-terminus of endolysin λ Sa2lys, has a two-fold higher in vitro antibacterial activity against streptococci than λ Sa2lys with a 3.8 and 1.8 log reduction in cell number after 30 min, respectively. Moreover, the enzymatic activity remains high under a broader range of salt concentrations and pH values in comparison to the native λ Sa2lys. The growth stage affects the activity of Art-240 less and a high activity was observed against stationary cells. A four- to twelve-fold higher dose of λ Sa2lys (12.5 nM of Art-240 vs. 150 nM for 2 log reduction) is needed to cause the same reduction than Art-240 to reach the same bactericidal effect as λ Sa2lys. The improved properties of Art-240 can be best explained by the positive charges of the PCNP, which acts as a local positive anchor for an enhanced binding of the Artilyns to the polyanionic cell surface (phosphate groups in teichoic acids) of Gram-positive bacteria.

4.4.2. Receptor-mediated uptake

Lukacik and his colleagues exploited the mode-of-action of pesticin to transfer an endolysin across the OM via receptor-mediated uptake. Pesticin is a bacteriocin produced by *Yersinia pestis* to kill closely related bacteria. It binds to outer membrane protein FyuA, followed by its transport across the membrane by the Ton transport system. Once pesticin has reached the periplasm, it will degrade the peptidoglycan layer, resulting in lysis of the competing bacteria (Lukacik et al., 2012a, 2012b; Patzer et al., 2012). Pesticin is a modular protein consisting of

two domains with dedicated functions: a N-terminal binding domain and a C-terminal EAD with muramidase activity. By solving the crystal structure of pesticin they deduced that N-terminal domain is essential for binding to FyuA. Producing *Y. pestis* strains express the pesticin immunity protein (Pim) that binds with the muramidase domain in order not to be killed by its own bacteriocin. A chimeric enzyme was constructed by exchanging the C-terminal muramidase for the bacteriophage T4 lysozyme, which is not recognized by Pim. Consequently, the chimeric enzyme lyses all strains with the same efficiency, more specifically it lyses all pathogens expressing FyuA, including uropathogenic *E. coli* (UPEC). Whereas a couple of point mutations in the binding region of FyuA could result in less or no recognition by the chimeric enzyme, this resistance mechanism may also reduce or eliminate the virulence of the resistant strain (Briers and Lavigne, 2015). A better understanding of the uptake mechanism and subsequent lysis will be necessary to reach clinical application and would broaden the applicability of receptor-mediated uptake against other pathogens (Lukacik et al., 2012b).

Yan et al. (2017) used a similar approach to enhance the antibacterial activity of Lysep3 against *E. coli*. Specifically, the N-terminal translocation and receptor binding domain of the bacteriocin colicin A of *E. coli* were fused to Lysep3. Colicin A exploits the Tol-Pal system to get access to the inner membrane where it inserts and forms a channel, causing membrane depolarization and subsequently cell death (Penfold et al., 2012). Colicin-Lysep3 is bactericidal against 22 out of 32 tested *E. coli* strains (≤ 0.5 log units), whereas it did not show any antibacterial activity against other Gram-negative bacteria (e.g. *P. aeruginosa*, *Salmonella* or *Shigella* Castellani). *E. coli* susceptibility likely depends on expression of the receptor. The mode-of-action of the endolysin was visualized by microscopic observations and electron microscopy: rod-shaped bacteria transform to oval-shaped cells when Colicin-Lysep3 is added, followed by rapid lysis within 15 min. The chimeric endolysin was evaluated in the digestive tract in a mouse intestinal infection model. Mice were fed with GFP expressing *E. coli* BL21 (DE3) cells and afterwards with Colicin-Lysep3. Small reduction in cell number were observed in the forepart of the intestine (11% reduction), followed by the posterior segment of the small intestine and the cecum (3% reduction) (Yan et al., 2017).

5. Preclinical analysis of endolysins

5.1. The unique properties of endolysins

Various independent in vitro and in vivo studies have extensively demonstrated the potential of endolysins or endolysin-based proteins as antibacterials. Since their conceptualization as antibacterial several strengths and weaknesses have been immediately recognized. Especially the proteinaceous nature of these antibacterials was novel and elicited unprecedented questions in terms of immunogenicity and stability, but many of these concerns have been addressed and resolved since then, resulting in successful (pre)clinical outcome (Table 1).

5.1.1. Specificity

A unique feature of endolysins is their specificity. Due to this specificity they will not disturb the normal microflora in contrast to broad-spectrum antibiotics (Nelson et al., 2012). Nevertheless, a narrow activity spectrum, as some endolysins are even serovar specific, could be a problem in polymicrobial infections or when the infection is not diagnosed at this specificity level. This can be solved by using an endolysin with a broader host spectrum or by engineering (e.g. mutagenesis or combining different endolysins domains) the lytic spectrum of the endolysin (Dong et al., 2015; Yang et al., 2014a).

5.1.2. Unique mode-of-action

As endolysins are enzymes, they have a unique mode-of-action against bacteria. They actively degrade the peptidoglycan, resulting in

fast osmotic lysis of the bacteria, whereas standard antibiotics rely on the inhibition of a metabolic step within the bacterial cell. The latter is fundamentally a much slower process. This is illustrated by the fact that it takes endolysins only 10 s to eliminate a cell culture of 10^7 bacteria to undetectable levels (Fischetti, 2003; Loeffler et al., 2001). Using real-time time-lapse microscopy an immediate lysis of *A. baumannii* upon exposure to Art-175 was observed (Defraigne et al., 2016). Moreover, the active mode-of-action makes it possible for endolysins to eliminate metabolically inactive cells such as persisters, which are often the source of reoccurring and chronic infections, while antibiotics do not affect them (Briers et al., 2014a; Gutiérrez et al., 2014).

5.1.3. Synergy

Antimicrobial synergy has been demonstrated for multiple combinations of endolysins with other endolysins, as well as with traditional antibiotics (Wittekind and Schuch, 2016). The observed synergistic effect between two or more endolysins with different catalytic specificity (e.g., chimeric construct of LysK and lysostaphin; Pal and Cpl-1; chimeric proteins of lysostaphin and HydH5, alone or in combination with LysH5, chimeric constructs of LysK, lysostaphin and Ami2638A, also combinations of the parental endolysins; chimeric endolysins with lysostaphin, B30 lysin and λ Sa2) is explained by an enhanced destructive effect when cleaving two different bonds at the same time within the three-dimensional PG meshwork. Alternatively, the cleavage of the first bond by one enzyme could result in better accessibility to the second target site by the other endolysin, causing a faster degradation of the substrate (Becker et al., 2008; Filatova et al., 2016; Loeffler and Fischetti, 2003; Rodríguez-Rubio et al., 2012; Schmelcher et al., 2012b, 2015a; Verbree et al., 2017a). The mechanism of synergy between endolysins and antibiotics remains unclear. Endolysins may enhance antibiotic activity in a similar way as PG cleavage could facilitate antibiotic uptake (e.g. LysABP-01 and colistin, Cpl-1 and gentamicin, MV-L and vancomycin, ClyS with vancomycin or oxacillin, P128 with gentamicin, vancomycin or ciprofloxacin, a CHAP-amidase chimeric endolysin derived from LysK and vancomycin). Specific combinations of an endolysin and an antibiotic can resensitize bacteria to antibiotics against which they are resistant (e.g. Cpl-1 and penicillin, MV-L and vancomycin). These combinations can reduce the emergence of antibiotic-resistant mutants and significantly reduces the required dose and duration of the treatment (Daniel et al., 2010; Djurkovic et al., 2005; Haddad Kashani et al., 2017; Nair et al., 2016; Rashel et al., 2007; Thummeepak et al., 2016).

5.1.4. Resistance development

Resistance development experiments in which strains were repeatedly exposed to subinhibitory concentrations of endolysins or Artilysins, failed to deliver resistant mutants in contrast to when strains were exposed to control antibiotics. The probability of resistance development against endolysins and Artilysins is therefore considered as low, especially when compared to traditional antibiotics (Briers et al., 2014a; Defraigne et al., 2016; Fischetti, 2005; Pastagia et al., 2011). One exception is the study of Becker et al. (2016). Serial exposure of *S. aureus* Newman to subinhibitory doses of endolysin LysK or lysostaphin resulted in a 42-fold and 585-fold increases in MICs in liquid culture, respectively. Resistance development could be eliminated or strongly reduced by the construction of two triple-acting chimeric fusions comprising three different EADs and one CBD (K-L and L-K; see Section 3.2), resulting in a 2-fold and 8-fold increase in MIC in liquid culture and no increase in MIC in plate lysis assays (Becker et al., 2016). Resistance development against the chimeric enzymatic P128 has also been reported by Sundararajan et al. (2014). However, the resistant isolate, became sensitive to β -lactam antibiotics, showed reduced in vitro growth rate and reduced in vivo virulence. Two other resistant isolates reverted to the original P128-sensitive phenotype. These observations suggest that P128-resistance comes at a fitness cost and hence a disadvantage in survival compared to non-resistant strains

Table 1

Strengths and weaknesses of endolysins. Different strengths and potential weaknesses related to endolysin-based antibacterials are summarized, specifically in view of their proteinaceous nature, along with the current corresponding state-of-the-art conclusions.

Strengths		Weaknesses	
Specificity	Do not disturb normal microbiota (Nelson et al., 2001)	Immunogenicity of proteins	Antibodies can be generated, but do not neutralize in vivo the action of endolysins, likely due to the strong binding of CBD and fast kinetics of endolysins that outcompete the action of antibodies (Jado et al., 2003; Loessner et al., 2002),
Mode-of-action	Fast, active degradation of peptidoglycan. Also, metabolically inactive cells (e.g. persisters) are lysed (Briers et al., 2014a; Gutiérrez et al., 2014)	Allergenicity	Not observed until now (Jun et al., 2017).
Synergy and resensitization	Synergism between endolysins with different catalytic specificities has been observed (Loeffler and Fischetti, 2003). Antibiotic-resistant bacteria become sensitive for the respective antibiotic if endolysin is added (Djurkovic et al., 2005).	Short half-life	Fast acting and strong binding can be sufficient (Jado et al., 2003; Loessner et al., 2002), some endolysins have a long half-life, several modifications are possible to extend life-time (Filatova et al., 2013; Resch et al., 2011a; Walsh et al., 2003).
Resistance development	Not observed for endolysins acting on the glycan chain and peptide stem, but for endopeptidases that cleave in the cross-bridge (lysostaphin) or between the cross-bridge and the stem peptide (CHAP domain of LysK and P128). Resistance development is associated with reduced virulence. Chimeric endolysins with triple acting EADs further lower the chance of resistance development (Becker et al., 2016; Fernández et al., 2017; Fischetti, 2005; Pastagia et al., 2011; Sundararajan et al., 2014).	Proinflammatory response due to cellular debris	Not observed by single or interval administration, though after continuous administration (Witzenrath et al., 2009; Zhang et al., 2016).
Post antibiotic effect	Subinhibitory concentrations of CF-301 have a strong effect on the (re)growth of bacteria. However, it still has to be confirmed as general feature for endolysins (Oh and Schuch, 2017; Schuch, 2016).	Intracellular bacteria are not accessible	Some native endolysins (PlyC) and chimeric endolysins (K-L) have the intrinsic ability to kill intracellular bacteria. Another option is to fuse protein transduction domains to endolysins to facilitate uptake in mammalian cells (Becker et al., 2016; Shen et al., 2016).

(Sundararajan et al., 2014). Notably, all (chimeric) endolysins to which resistance or a lowered susceptibility has been observed, act on the pentaglycine bridge of *S. aureus* peptidoglycan. Both the CHAP domains of LysK and P128 cleave between D-alanine of the stem peptide and glycine of the pentaglycine cross-bridge (Bastos et al., 2010; Becker et al., 2009). Resistance against lysostaphin can arise through shortening of the pentaglycine bridge to one or three glycine residues, or replacing individual glycines by serines (Climo et al., 1998; DeHart et al., 1995; Grundling et al., 2006). This resistance mechanism is associated with an increased susceptibility for β -lactam antibiotics and results in less virulent and resilient bacteria (Climo et al., 2001; Kusuma et al., 2007). The similarity in loss of fitness between lysostaphin- and P128-resistant mutants suggests that they may have a similar resistance mechanism. Both lysostaphin- and P128-resistant isolates are mutated in the *femA* gene (pentaglycine synthetase). However, the frequency of resistance development was calculated to be lower against P128 (3.5×10^{-6} to 2×10^{-7}) than against lysostaphin (5.3×10^{-1} to 1.0×10^{-7}). Also, altered pentaglycine cross-bridges (GGSGG, AGSGG) that evoke lysostaphin-resistance were able to be cleaved by P128. This shows that in spite of sharing a common mechanism, P128 is capable of inhibiting certain lysostaphin-resistant staphylococcal variants (Sundararajan et al., 2014). Also the chimeric enzymatic SsaALP-LBD (fusion between the CHAP domain of SsaALP and the SH3b domain of lysostaphin) is able to effectively kill lysostaphin-resistant *S. aureus* (Osipovitch et al., 2015). A two-fold reduced susceptibility has been reported for the chimeric endolysin CHAP-SH3b (CHAP domain of the VAPGH of *S. aureus* bacteriophage vB_SauS-phiPLA88 (HydH5) and the SH3b domain of lysostaphin). This reduced susceptibility was caused by transcriptional downregulation of autolysins such as AtlA (Fernández et al., 2017) and thus follows a different resistance mechanism. Endolysin(-based antibacterials) cleaving the glycan chain or the amide bond between MurNAc and the stem peptide are therefore the best candidates in terms of resistance development, since the cross-bridge is highly variable among Gram-positive bacteria and can be easily changed.

5.1.5. Immune response

A potential concern related with the proteinaceous nature is an adverse immune response by the generation of neutralizing antibodies (Fischetti, 2010). Experiments with endolysin Cpl-1 in immunized rabbit serum (in vitro) and immunized mice (in vivo) showed that although antibodies were produced, they are not able to completely inactivate the endolysin or have no significant effect at all (Loeffler et al., 2003). Similar results were found with other endolysins (Jado et al., 2003; Rashel et al., 2007; Zhang et al., 2016). These findings suggest that the immune response is not able to neutralize the endolysins, which could be explained by the strong binding of the CBD to its substrate and the fast enzyme kinetics outcompeting the hosts immune response (Jado et al., 2003; Loessner et al., 2002). In addition, no severe allergic reactions have been reported as adverse events in (pre-) clinical studies including two separate phase I studies with SAL200 (ClinicalTrials.govNCT01855048, published in (Jun et al., 2017)) and CF-301 (ClinicalTrials.govNCT02439359 presented in (Cassino et al., 2016)), respectively.

5.1.6. Post antibiotic effect

ContraFect has reported a post antibiotic effect (PAE) for *S. aureus* endolysin CF-301. The PAE is the time period during which there is delayed regrowth of bacteria when administration of the antibiotic has ceased and serum concentrations have fallen below the MIC (MacKenzie and Gould, 1993). Initial tests with CF-301 against 14 staphylococcal isolates in human serum, showed a durable PAE of 4.8 h and a post-antibiotic sub-MIC effect (PA-SME) of up to 7.8 h. A synergistic effect was seen when daptomycin was added. Moreover, sub-inhibitory concentrations of CF-301 ($0.004 \times \text{MIC}$) resulted in an increased antibiotic susceptibility, decreased biofilm formation and reduced growth rates (in vitro and in vivo) due to membrane damage, even when they have not been previously exposed. This is called the sub-MIC effect or SME. Finally, the in vivo PAE in mice is significantly longer, reaching 23–26 h. It remains to be confirmed if the observed effects of CF-301 are a general feature for endolysins or specific for CF-301 (Oh and Schuch, 2017; Schuch, 2016).

5.1.7. Half- and shelf-life

Generally, endolysins have a short half-life in blood, approximately between 4 and 40 min depending on the endolysin (Jun et al., 2017; Loeffler et al., 2003). Nevertheless, some endolysins apparently have a longer half-life (e.g. CF-301: 11.3 h) (Cassino et al., 2016). Moreover, the half-life is species-dependent: e.g. the half-life of endolysin SAL200 varies between 2 min (in humans) to 9 h (in monkeys) (Jun et al., 2016, 2017). The short half-life is expected not to be a problem, since endolysins act fast and are repeatedly shown to be effective in diverse animal models. Notwithstanding that the clearance time depends on the endolysin and species, it limits their window of action and the endolysin will therefore have to be administered repeatedly (e.g. interval injection) (Entenza et al., 2005). Modification with polyethylene glycol (PEG), PEGylation, is a generally used approach to improve the pharmacokinetics of biomolecules, including extending the half-life of proteins. PEGylation has been successfully shown for lysostaphin, extending the half-life from < 1 h to 24 h (Walsh et al., 2003). Yet, when applied to the Cpl-1 endolysin, a complete loss of its in vitro antibacterial activity was observed, likely due to interference of the bulky PEG with the mode-of-action of the endolysin. PEGylation must thus be evaluated for endolysins in an empirical way (Resch et al., 2011b). In contrast, dimerization by the introduction of a disulphide bond is an efficient approach to increase the half-life of Cpl-1. Cpl-1 homodimers have a two-fold higher in vitro activity and ten-fold decrease in plasma clearance (Resch et al., 2011a). Another important aspect of pharmaceutical formulations is their stability during storage or the shelf-life. One approach to stabilize endolysin is based on complexation with block copolymers of polycationic polymers (poly-L-lysines (PLL) with polyethylene glycol). This has been demonstrated for LysK. Complexation did not alter the specificity, but the LysK complexes have increased stability at physiological temperature and a 10–50 times longer shelf-life were reported after complexation. In addition, the LysK complexes have a 3–3.5 times higher in vitro antibacterial activity than the native endolysin. It was hypothesized that the electrostatic interactions between the cationic polymers and negative charged teichoic acids of the bacterial membrane influenced the in vitro activity of LysK (Filatova et al., 2013). The same authors demonstrated that a triple-acting lytic chimera, K-L, can be incorporated in nanoparticles of block-copolymers of poly-L-glutamic acid and polyethylene glycol. This immobilization does not interfere with the enzyme stability or activity and could potentially reduce the immunogenicity upon systemic admission (Filatova et al., 2016).

5.1.8. Inflammatory response

The release of proinflammatory cellular debris ((lipo)teichoic acids, lipopolysaccharides and peptidoglycan) is inherently related to the mode-of-action of endolysins (but also to other cell wall acting antibiotics). A too extensive inflammatory response could lead to life-threatening complications, such as septic shock and multiple organ failure (Nau and Eiffert, 2002). Initial work points at the importance of the administration mode. A recent study by Zhang et al. (2016) used a single intravenous injection of endolysin LysGH15 to protect mice against lethal infection with MRSA. The concentration of pro-inflammatory cytokines declined significantly and the examined organs (heart, liver, spleen, lung, kidney, and colon) showed no significant inflammation or other pathological changes after LysGH15 treatment. This supports the observations made by Witzernath et al. in 2009 in a different animal model. Here, the endolysin Cpl-1 was administered by intraperitoneal injections in intervals of 12 h, resulting in reduced focal inflammation in the lungs compared to untreated animals. Similar observations were done with in vitro human bronchial epithelial cell layers (Witzernath et al., 2009). On the contrary, a continuous intravenous infusion of Cpl-1 leads to an increase of the cytokine concentration (Entenza et al., 2005). A possible explanation for the differences in inflammatory response can be found in the enhanced fragmentation of the bacterial cell wall when high doses of endolysin

are used, e.g. with during continuous administration (Fischetti, 2010). These findings indicate that an adverse response can be avoided if the correct dosing, e.g. single or interval injection, is used.

5.1.9. Intracellular pathogens

Many pathogens reside intracellularly to evade the host immune system and are thus more difficult accessible for antibacterials. Fusions of endolysins with protein transduction domains (PTDs) were explored to enable passage of the endolysin across the eukaryotic membrane. However, two independent studies show that chimeric endolysins (Becker et al., 2016) and a native endolysin (Shen et al., 2016) are capable to enter mammalian cells without a PTD as well. Becker et al. (2016) constructed the chimeric endolysins K-L and L-K (see Section 3.2) and several PTD sequences were fused to their C-terminus. Several cell types that can be invaded by *S. aureus* (bovine mammary epithelial cells, murine osteoblasts and human brain microvasculature endothelial cells) were tested. Both K-L and K-L-PTD can kill intracellular *S. aureus*, thus surprisingly, a PTD is not required for intracellular killing. In contrast, wildtype L-K and L-K-PTD are not able to kill intracellular *S. aureus*. In an in vivo mastitis model, both K-L and K-L-PTD reduce the bacterial cell number, but the latter reduces the cell number by a three order of magnitude more. Further research has to be done to explain the differences between the ex vivo and in vivo assays and the difference in activity between L-K and K-L as their sequence is almost identical. The authors suspect that the 3D-conformation of the domains plays a crucial role for efficient uptake through the eukaryotic membrane (Becker et al., 2016). In another study, the authors also anticipated that the endolysin, PlyC, would need to be modified with PTDs to be able to kill intracellular *Streptococcus pyogenes* (Spy). Yet, 50 µg/mL PlyC was able to reduce 90–95% of CFU of intracellular Spy in A549 epithelial cells within 1 h. The authors demonstrated via mutagenesis of Arg and Lys residues to Glu that cationic amino acids at the surface of the PlyCB subunit are essential for internalization of PlyC, without being toxic for eukaryotic cells. Via a phosphoinositide array it was demonstrated that PlyCB specifically interacts with anionic phospholipids (phosphatidylinositol, phosphatidic acid and phosphatidylserine). Furthermore, it was demonstrated via modeling of phospholipids binding that mutant PlyCB_{R66E}, which lost its binding to Spy and internalization activity, is crucial for interactions with the phospholipid head groups. The internalization of PlyCB is an energy- and lipid-raft-dependent process similar to caveolae-mediated endocytosis, which is suggested as the internalization pathway. Additional research is needed to clarify the intracellular transport of PlyC and its interactions with Spy. Nevertheless, fusion of PlyCB to other endolysins could make it possible to target other bacterial intracellular pathogens (Shen et al., 2016).

5.2. Administration routes of endolysins

Bacteria can colonize and infect almost any site in the human body. Therefore, it is important that endolysins can reach the site of infection. However, an adequate and safe manner of administration of endolysin is still a largely unexplored area (Hathaway et al., 2017b). Injection of endolysins has been studied as a major administration route since the early enzybiotic research. Today, several types of injection have proven their efficacy in animal models: intravenous (Entenza et al., 2005; Loeffler et al., 2003), intravitreal (Singh et al., 2014), intraperitoneal (Daniel et al., 2010; Gilmer et al., 2013; Jado et al., 2003; Lood et al., 2014, 2015; Schuch et al., 2002) and intracisternal (Grandgirard et al., 2008). Moreover, SAL200 and CF-301 are both currently in a clinical Phase II trial (Table 2) and have been formulated for intravenous injection (Jun et al., 2014). Injection has been mainly evaluated for the treatment of systemic infections such as meningitis, endocarditis, osteomyelitis and bacteremia in animal models.

For more accessible topical bacterial infections, several options of administration have been evaluated in animal models or case studies with animals. P128 is a chimeric enzybiotic with anti-staphylococcal

Table 2
Main companies and their clinical research.

Company	Market	Main endolysin(s)	Target	Clinical trial(s)	Identifier
Contrafect	Health-care	CF-301	<i>S. aureus</i> bacteremia	Starting Phase II	NCT03163446
Gangagen	Health-care	P128	<i>S. aureus</i> in nasal settings	Finished Phase IIa	NCT01746654
Intron Biotechnology	Health-care	SAL200 (commercial name N-Rephasin®)	Staphylococcal infections	Starting Phase IIa	NCT03089697
Microeos	Cosmetics and (entering) health-care	Gladskin (Staphetek™ XDR.300)	Staphylococcal infections	Starting combined Phase I and II	NCT02840955
Lysando	Wound care	Medolysin®	Bacterial wound infections	(Pre)clinical	N.A.

activity (see Section 3.2) and has been formulated in a hydrogel (Vipra et al., 2012). The hydrogel consists of a mixture of hydroxyethyl cellulose, propylene glycol and glycerin. The P128 hydrogel had an in vitro 2–4 log reduction on the cell number of nasal staphylococcal isolates in their physiological condition, whereas the native P128 had a 4 log reduction (in vitro) on six MSSA and MRSA strains (Vipra et al., 2012). Abouhmad et al. (2016) fused T4 lysozyme to a cellulose binding module of *Cellulomonas fimi* for immobilization of the endolysin on cellulosic gauze material, which is typically used for wound dressings. The fusion protein retained its antibacterial activity against Gram-positive and -negative pathogens, while being irreversibly bound to the wound dressing gauze. Moreover, the immobilized protein has a higher thermostability and shelf-life when compared to the free endolysin. In a follow-up study, the same authors showed that immobilization of the T4 lysozyme on cellulose nanocrystals further enhances the antibacterial activity and shelf-life (Abouhmad et al., 2017).

An advanced mode-of-administration is the local release of endolysin at the site of infection to ensure a maximal possible therapeutic effect and to diminish possible side effects. If this release is coupled to an external trigger (e.g. temperature, pH, light, ultrasound or biomarker signals) this is called a stimuli-responsive system (Bean et al., 2014; Mura et al., 2013). Such a system has been developed by Hathaway and colleagues for a synergistic mixture of CHAP_K and lysostaphin against *S. aureus* (Hathaway et al., 2017a). They exploited the properties of the thermoresponsive polymer poly(*N*-isopropylacrylamide) or PNIPAM. The PNIPAM particle will collapse at a certain release temperature (lower critical solution temperature or LCST), which results in the expulsion of its cargo. The authors have previously optimized the LCST to 34 °C, a biologically relevant temperature for wound infection, by including allylamine. The nanoparticles were attached to fabric swatches of nonwoven polypropylene via plasma activation, simulating a wound dressing or bandage. If this system was exposed to elevated temperatures (above 34 °C), a specific antibacterial effect of > 4 log units was observed, whereas < 1 log reduction was observed at lower temperatures (Hathaway et al., 2017a, 2017b).

The respiratory system can be infected by several bacteria leading to different diseases ranging from a streptococcal pharyngitis to life-threatening pneumoniae caused by *S. pneumoniae* (Arroll, 2011; Doehn et al., 2013). Doehn et al. investigated aerosols as formulation. A single dose of aerosolized Cpl-1 rescues mice from fatal pneumococcal pneumonia (Doehn et al., 2013). The aerosol treatment efficiently reduces the bacterial load in the lungs, protects the mice from pneumococcal induced bacteremia and leads to a significant increase in survival rate. Although the inhalation of Cpl-1 initially leads to an increase of inflammatory cytokines, these symptoms recede and no adverse effects are permanent.

Bacteria can cause several gastrointestinal diseases (e.g. bacterial gastroenteritis, bacillary dysentery, pharyngitis, cholera, etc.) and some of these diseases can be life-threatening. Treatment of those diseases is often challenging, especially with a proteinaceous agent, due to the harsh conditions (acidic and basic pH, bile salts, proteases, etc.) that are present in the gastro-intestinal (GI) tract (Hathaway et al., 2017b). Using an engineered probiotic microbe that secretes an endolysin at a specific site can be a possible route for treating GI associated diseases.

Gervasi and colleagues demonstrated the potential of such a system in two studies. Specifically, they engineered *Lactobacillus johnsonii* to express and secrete the endolysin CP25L, which has a specific antibacterial activity against *Clostridium perfringens* (Gervasi et al., 2014a, 2014b). In an initial study, they demonstrated that *L. johnsonii* secretes CP25L extracellularly and showed that CP25L has in vitro antibacterial activity in a medium that simulated the GI tract. A plate assay with autoclaved *C. perfringens* confirmed that the engineered strain is able to produce and lyse the targeted bacterium (Gervasi et al., 2014a). In a follow-up study, the authors investigated whether *L. johnsonii* can survive, secrete and kill *C. perfringens* in GI simulated settings (batch fermenter). Killing has been proven twice, however, a third repeat was unsuccessful. It was hypothesized that this lack of robustness is caused by the fact that the probiotic strain needs to be present in high numbers for effective pathogen control.

Recently, a paper took the concept of the stimuli-responsive system a step further for treatment of GI infections by *Vibrio cholerae*. *E. coli* was engineered to a sense-and-kill system with a synthetic gene circuit that specifically detects CAI-1, a quorum sensing (QS) molecule solely used by *V. cholerae*. If this QS molecule is detected, *E. coli* will start expression of two proteins: YebF-Art-085 and Art-085. Art-085 does not kill *E. coli* from within, but when YebF, a secreted protein, is fused to the N-terminus of Art-085, the fusion protein leads to fast and efficient cell lysis. Upon cell lysis, native Art-085 will be released and will effectively kill *V. cholerae* up to an 8 log reduction, reducing *V. cholerae* cell numbers below 100 CFU/mL within 1 h. In sum, all previous studies show that there are a multitude of systems under development to target any bacterial infection site. However, many of these administration routes need to be further optimized before they can be evaluated in clinical settings (Jayaraman et al., 2017).

6. Endolysins in the clinical stage

SAL200 (commercial name: N-Rephasin®, developed by Intron Biotechnology) is the formulated, recombinant variant of endolysin SAL-1, which was derived from the staphylococci infecting phage SAP-1 and is highly similar to two other well-characterized staphylococcal phage endolysins, LysK and LysGH15 (Gu et al., 2011; Jun et al., 2011). SAL-1 is formulated using 0.01 M L-histidine (pH 6.0), 5% (w/v) sorbitol, 10 mM CaCl₂ and 0.1% (w/v) Poloxamer 188. Calcium ions are added, as they enhance the enzymatic activity. They also reduce the stability of the endolysin, but this effect is countered by including Poloxamer 188, a non-ionic copolymer of polyoxyethylene and polyoxypropylene. The potency of the formulated SAL200 as an anti-staphylococcal agent is shown in vitro against various clinical *S. aureus* isolates, a biofilm model and in vivo by intravenous injection in a mouse challenge model (Jun et al., 2013). Jun et al. reported a GLP-compliant study on rats and dogs, including general toxicology and safety pharmacology tests for SAL200 in 2014. Both, single and repeated-dose, toxicity test in rats yielded no adverse events or laboratory abnormalities for all dosing levels. Similar results were obtained in the repeated dose experiments in dogs. Yet, clinical signs (subdued behavior, prone position, irregular respiration and vomiting) were observed in dogs after additional injections, nevertheless all signs disappeared

within 1 h. Similar transient changes in cardiovascular function were seen upon the third injection and again the signs resolved after 6 h. Regarding pharmacologic safety, no adverse effects were observed in the central nervous and respiratory systems. Finally, it was determined that specific antibodies against SAL200 could be raised in both rats and dogs, and their concentration increased towards the end of the study (Jun et al., 2014).

After these initial preclinical tests, the pharmacokinetics of SAL200 were determined on a primate model (Jun et al., 2016). No serious adverse events or laboratory abnormalities were detected in both the single dose escalation or interval intravenous injection of SAL200 in a phase I study (clinicaltrials.gov/NCT01855048 and reported in (Jun et al., 2017)). As expected antibodies could be raised against endolysins and the level was proportional to the administered dose (ranging from 2 to 12 µg/mL). Analysis of the pharmacokinetics and pharmacodynamics indicated the therapeutic potency of SAL200. The antibacterial activity in blood was determined 1 h after injection by spotting the withdrawn blood on a lawn of *S. aureus* (ex vivo blood assay). The formed halos were qualitatively compared to halos formed by a standard series of SAL200. Dosing regimen of > 1 mg/kg of SAL200 seems to be a viable treatment option according to the authors. This was based on the following observations: (i) the residual SAL200 concentration after 1 h was higher than 0.078 µg/mL, which is the minimum bactericidal concentration for 1×10^6 CFU/mL in a serum environment; (ii) the time needed to reduce the initial bacterial concentration was < 10 min (Jun et al., 2017). Based on these data, the safety and efficacy of SAL200 can be tested in humans with *Staphylococcus aureus* bacteremia. Recently, a phase II clinical trial (clinicaltrials.gov/NCT03089697) has been started.

The New York-based company ContraFect has entered clinical trials with endolysin CF-301. This endolysin is also referred to as PlySs2, which was identified in a prophage of *Streptococcus suis* and has a potent anti-staphylococcal activity (Gilmer et al., 2013). The FDA granted CF-301 Fast Track designation. ContraFect released their clinical data. The safety and tolerability of single intravenous doses of CF-301 was tested in a phase I clinical trial on healthy human subjects (clinicaltrials.gov/NCT02439359). Participants received a placebo or a 2-h intravenous infusion of CF-301 with doses between 0.04 and 0.4 mg/kg/dose. CF-301 was reported by ContraFect to be well tolerated with no clinical adverse safety signals (hypersensitivity or serious adverse events). Specific anti-CF-301 antibodies did develop, but no correlation with immunoglobulin E and ex vivo basophil activation was observed (Cassino et al., 2016; Jandourek et al., 2017a). Additional inflammatory markers, erythrocyte sedimentation rate, high-sensitivity C-reactive protein, complement factors (Bb, C3a, C5a, and CH50), IL-6 and TNF-α were further investigated, but no clinical relevant changes were measured (Jandourek et al., 2017b). Based on PK/PD models from animal studies and the data of the phase I study, a dose of 0.25 mg/kg of CF-301 could be sufficient to treat *S. aureus* infections in humans without inducing adverse events (Cassino et al., 2016; Ghahramani et al., 2017; Rotolo et al., 2016). A clinical Phase II trial has been initiated to evaluate the safety, efficacy and pharmacokinetics of CF-301 in addition to standard of care antibacterial therapy for treatment of *S. aureus* bacteremia (clinicaltrials.gov/NCT03163446).

P128 is the only engineered enzymatic (chimer) that is currently in the clinical development stage and was developed by the Indian company Gangagen. Specifically, P128 was developed for the intranasal use against *S. aureus*, including MRSA. In initial experiments, P128 (10 µg/mL) displayed a strong in vitro antibacterial activity (> 99% reduction of CFU) on several clinical isolates of *Staphylococcus* strains, including MRSA. Moreover, P128 was formulated as a hydrogel and tested in vivo in a nasal rat model, where it could decolonize four of nine animals completely. In general, P128 treated animals had a 2 log reduction in cell number compared to the controls (Paul et al., 2011). The efficiency of the P128 hydrogel was further evaluated for the treatment of canine staphylococcal pyoderma, a skin problem often found in dogs. A 5–6 log

reduction on isolates of canine pyoderma was observed in in vitro efficiency tests with the P128 hydrogel, irrespective of the presence of antibiotic resistance mechanisms. The in vivo efficiency of the P128 hydrogel was tested on 17 adult dogs of several breeds that were diagnosed with pyoderma. All dogs were cured and no recurrence of pyoderma was observed after two months, the end of the study (Junjappa et al., 2013). P128 exerts potent antibiofilm activity on sinus-derived *S. aureus* biofilms (Drilling et al., 2016) and biofilms of Coagulase-Negative staphylococci can be eradicated with P128 (2–5 log reduction in cell number). Moreover, P128 shows synergistic effects with other antibiotics (gentamicin, vancomycin or ciprofloxacin) in treating staphylococcal strains (Poonacha et al., 2017). No symptoms of systemic anaphylaxis (type I hypersensitivity) or P128-related tissue injuries (type III hypersensitivity) were seen in any of the P128-dosed animals. As seen in other preclinical tests of endolysins, P128-specific antibodies could be raised, but remained at low concentrations. The efficacy of P128 for systemic infections was demonstrated for MRSA bacteremia with a renal abscess rat model. A single dose of 2.5 mg/kg of P128 remained for 15 min above the MIC (4 µg/mL). P128 was able to rescue rats from a lethal dose of MRSA following a single intravenous injection (Channabasappa et al., 2017). The pharmacokinetics and efficacy of P128 were further investigated in a standard neutropenic mouse model for systemic MRSA infection using a single intravenous dose. Maximum efficiency was seen 30 min after injection, indicating a fast antibacterial effect. Cell numbers remained low for 24 h after ceasing the treatment, although the concentration of P128 in circulation was below the MIC. This could indicate a prolonged antibacterial effect or PAE, as seen for CF-301, but additional tests are needed to confirm these data. A long half-life of 5.2 and 5.6 h was observed for the highest dose, 30 and 60 mg/kg, respectively (Sriram et al., 2017). The clinical trials register mentions that a combined phase I and phase II clinical trial (clinicaltrials.gov/NCT01746654) has been completed, but no final data have been reported to date.

Staphefekt™, the active compound of the cetomacrogol-based cream and gel Gladskin, was launched in 2013 by the Dutch company Microcos as a (class 1) medical device and is sold as an over-the-counter product for human use for inflammatory skin conditions such as eczema, rosacea and acne (Totté et al., 2017). A recent case study on three human subjects with *S. aureus*-related skin conditions, folliculitis and impetiginized dermatitis showed that Staphefekt™ improved the clinical symptoms, but they rapidly recurred if the treatment was stopped. Probably this is due to the recolonization of *S. aureus* from the nose and environment. Clinical trials (clinicaltrials.gov/NCT02840955) to evaluate the safety and efficiency of Staphefekt™ have been announced (Totté et al., 2017).

The company Lysando has announced the development of Medolysin®, a wound care spray based on Artilysin® with bactericidal activity (Briers et al., 2014a, 2014b). In addition, the Medolysin® wound spray creates an optimum moisture and a protective film to support wound healing. Lysando reports that the wound healing process started immediately after application for 90% of the patients, resulting in a reduction of the wound sizes. Complete healing of the wounds is described for 40% of the patients within 30 days. A coma patient with severe decubitus and chronically infected by MRSA showed complete wound healing after ten months (Lysando, 2017).

7. Conclusion

The endolysin research field has undergone a significant acceleration in the last decade. This progress has now yielded a multitude of (engineered) endolysins against many major Gram-positive and Gram-negative pathogens. Different companies are developing endolysins against Gram-positive and Gram-negative pathogens and have reported on the preclinical or clinical progress of their lead products in the development pipeline (Table 2). Simultaneously, extensive engineering efforts have taught us that the modular architecture of endolysins

provides us with a practically infinite number of possibilities to customize the properties of these enzyme-based antibacterials. In addition, single domains of endolysins are increasingly used for other applications such as diagnostics and immune-based therapeutics. There is still a vast sequence space of variants that can be imagined towards novel enzybiotics or other applications. In sum, the synthetic biology of modular endolysins is still in its infancy, but has shown us already some exciting glimpses for the future.

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