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Research review paper

Design strategies for positively charged endolysins: Insights into Artilysin development

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ABSTRACT

Endolysins are bacteriophage-encoded enzymes that can specifically degrade the peptidoglycan layer of bacterial cell wall, making them an attractive tool for the development of novel antibacterial agents. The use of genetic engineering techniques for the production and modification of endolysins offers the opportunity to customize their properties and activity against specific bacterial targets, paving the way for the development of personalized therapies for bacterial infections. Gram-negative bacteria possess an outer membrane that can hinder the action of recombinantly produced endolysins. However, certain endolysins are capable of crossing the outer membrane by virtue of segments that share properties resembling those of cationic peptides. These regions increase the affinity of the endolysin towards the bacterial surface and assist in the permeabilization of the membrane. In order to improve the bactericidal effectiveness of endolysins, approaches have been implemented to increase their net charge, including the development of Artilysins containing positively charged amino acids at one end. At present, there are no specific guidelines outlining the steps for implementing these modifications. There is an ongoing debate surrounding the optimal location of positive charge, the need for a linker region, and the specific amino acid composition of peptides for modifying endolysins. The aim of this study is to provide clarity on these topics by analyzing and comparing the most effective modifications found in previous literature.

1. Introduction

Bacteriophages or phages are viruses that specifically infect bacteria. Their action was first described in 1915 by Frederick Twort (Twort, 1915). However, their use as a biocontrol agent for treating bacterial infections in animals and humans was introduced later by Felix d'Herelle (Salmond and Fineran, 2015), marking the starting point of phage therapy. While certain treatments have demonstrated efficacy, the lack of consistent results and the advent of antibiotics relegated phage therapy to the background. Nevertheless, with the rise of antimicrobial resistance (AMR) and the need to develop antimicrobial therapies other than antibiotics, the interest in phages and phage-encoded products have been renewed.

As viruses, bacteriophages are obligate parasites that require a bacterial host to replicate and multiply. A typical infection cycle involves several defined steps, initially, phages are attached to the host cell surface through the interaction of specific receptors and, if present, tail spikes of phage external structure. At the onset of infection, structural proteins, also termed virion-associated lysins (VALs), locally degrade the peptidoglycan in the bacterial cell wall from the outside. This degradation facilitates the insertion of the virus genetic material into the cell (Fig. 1 A). Subsequently, a new viral progeny is assembled using the host cell machinery and at the end of the cycle, a set of different proteins are synthesized to participate in the coordinate disruption of the bacterial cell wall (from within) allowing the release of the newly assembled virions. Among these proteins, phage-encoded peptidoglycan hydrolases or endolysins participate in the enzymatic degradation of the bacterial peptidoglycan, which leads to the rupture of the cell wall and consequently bacterial death. However, endolysin access to peptidoglycan is limited by the presence of the inner membrane. To gain access to the

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peptidoglycan layer, most endolysins require the action of another phage protein named holin. When holin concentration in the cytoplasmatic membrane reaches a threshold level, individual subunits oligomerize into pore forming structures [3]. This process facilitates the subsequent access and degradation of peptidoglycan by endolysins, which occurs from this point onward (Fig. 1 B).

1.1. Endolysins

Although endolysins have evolved to promote peptidoglycan degradation from inside, this lytic activity also occurs when these proteins are applied exogenously to the bacterial cells. For that, endolysins are recombinantly produced using different bacterial expression systems. Among these systems, *Escherichia coli* strains are the most popular choice due to their ease of genetic manipulation and well-established protocols. However, other cloning hosts, such as *Lactococcus lactis,* can also be used for this purpose (Chandran et al., 2022; Gaeng et al., 2000; O'Flaherty et al., 2005). The choice of the host organism ultimately depends on the specific requirements of the experiment and downstream applications. Based on the cell envelope structure, Gram-positive bacteria are especially sensitive to the external application of endolysins due to the lack of an outer cell membrane. However, some studies have demonstrated that the presence of wall teichoic acids (WTAs) hinders the enzyme's ability to bind to cells and effectively cleave the peptidoglycan within the cell wall (Eugster and Loessner, 2012; Gouveia et al., 2022; Wu et al., 2016). In contrast, Gram-negative bacteria, with their outer membrane, may limit or completely block their action (Fig. 2).

Numerous studies have highlighted the potential of endolysins as antimicrobial agents and their potential applications in clinical settings. Endolysins, as phage-derived elements, offer at least two advantages compared to their phage counterparts. Firstly, endolysins tend to exhibit a broader host range than phages, which typically target only a single species or even specific strains within a given species (Hyman and Abedon, 2010). Secondly, bacteria have developed a complex arsenal of anti-bacteriophage strategies, which could potentially lead to resistance through various mechanisms (Hyman and Abedon, 2010). In contrast, several studies have indicated that resistance to endolysins occurs at very low levels (Dams and Briers, 2019). Therefore, to overcome these limitations, phage therapy usually requires the use of phage cocktails that are specifically developed for each patient (Gill and Hyman, 2010).

On the other hand, compared to broad-spectrum antibiotics, the application of endolysin as antibacterial agents provide several advantages: (1) Specificity: endolysins are highly specific with little to no effect on non-target bacteria (Abdelkader et al., 2019), in contrast, antibiotics can also target beneficial bacteria, leading to various adverse effects; (2) Modularity: through protein engineering, endolysins can be easily manipulated to generate novel endolysin variants with improved specificity, potency, and stability (Kashani et al., 2017); (3) Resistance: different studies have demonstrated that the chance of developing antimicrobial resistance towards endolysins is much reduced compared to that observed for antibiotics (São-José, 2018). Additionally, the mechanisms that provide resistance to endolysins seems to pre-exist in bacterial species and does not readily emerge de novo (Grishin et al., 2020). Also, these mechanisms mostly rely on the modification of peptidoglycan which seriously compromises bacterial fitness and virulence (Kusuma et al., 2007). Additionally, several endolysins are currently undergoing successful clinical trials for the treatment of bacterial infections, with promising results observed for Exebacase (lysin CF-301) (Fowler et al., 2020), SAL200 (Jun et al., 2017), and XZ.700 (Kuiper et al., 2021). These advancements are paving the way for potential approval and commercialization of lysin therapy.

1.2. Architecture of endolysins

Even targeting the same bacterial structure, endolysins are diverse in their structure, catalytic activity, specificity, and enzyme kinetics. This diversity arises from the various chemotypes of peptidoglycan found in bacterial species, as well as the wide range of secondary carbohydrate

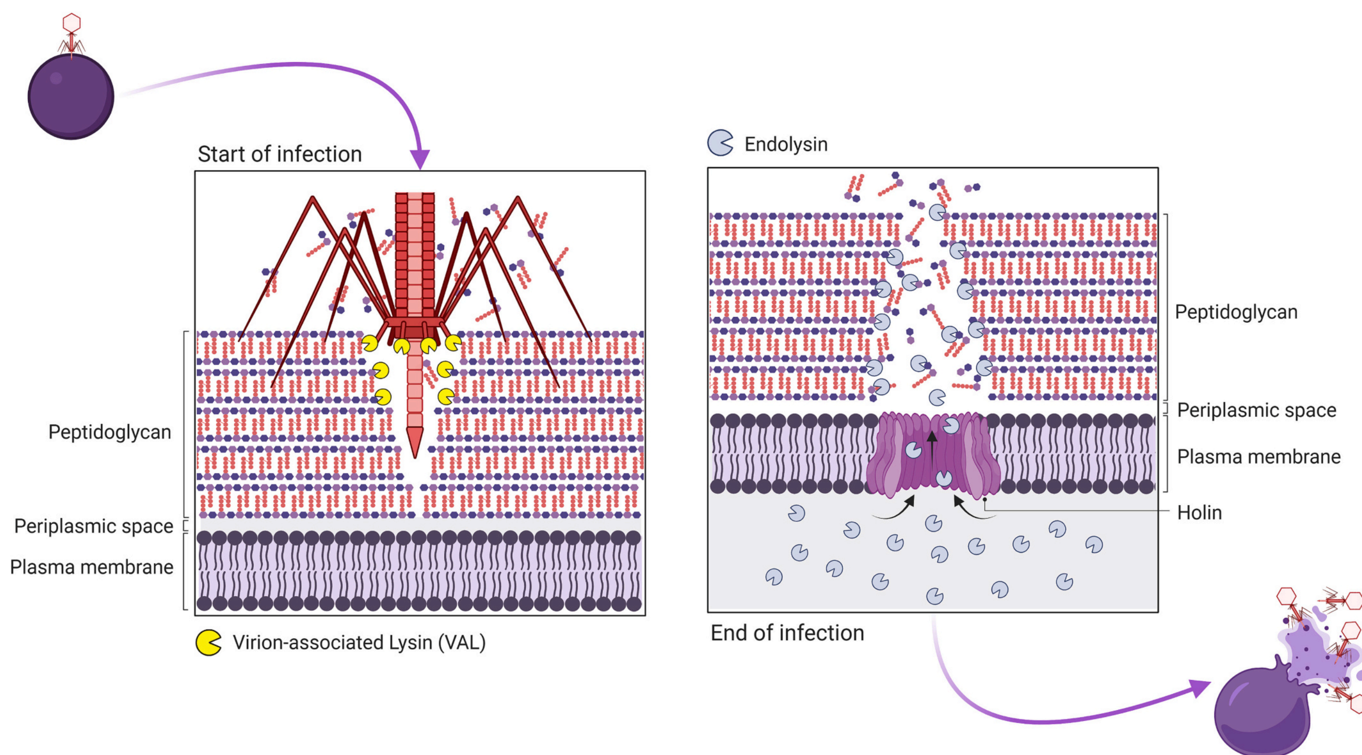

Fig. 1. Bacterial cell wall degradation by phage enzymes. At the beginning of phage infection cycle, virion-associated lysins (VALs) digest bacterial peptidoglycan, allowing the phage tail to penetrate the bacterial cell wall. At the end of phage infection cycle, holin proteins create pore-forming structures that perforate the bacterial plasma membrane, enabling endolysins to access the peptidoglycan layer.

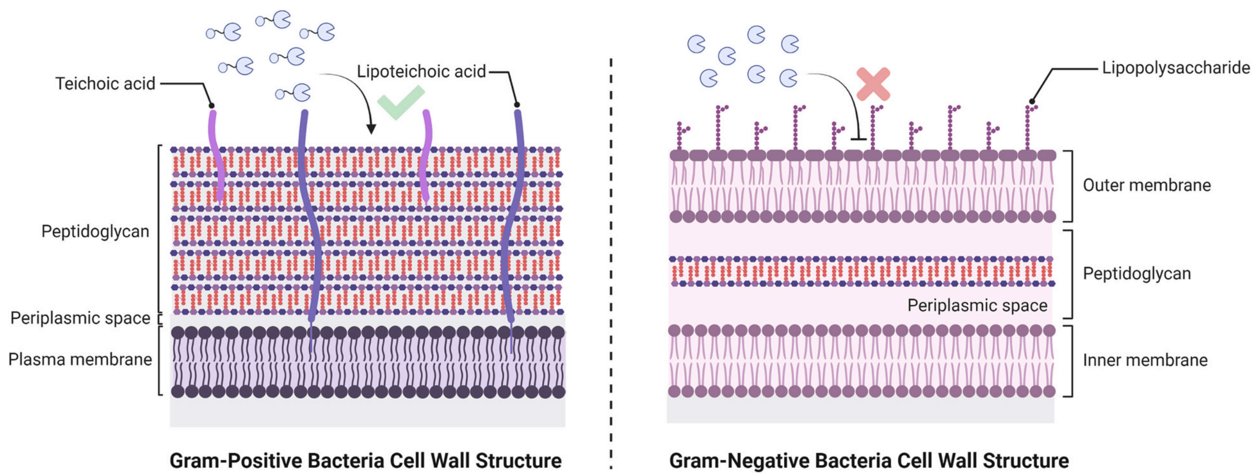

Fig. 2. Effect of the external addition of endolysins on different cell wall structures. In Gram-Negative bacteria, the presence of an outer membrane limits the effectiveness of endolysin treatment. On the other hand, Gram-positive bacteria lack the protective outer cell membrane, facilitating the access of endolysins to the peptidoglycan layer and resulting in higher peptidoglycan-disrupting activities upon external addition.

polymers associated with cell walls (Schleifer and Kandler, 1972). In the case of Gram-positive bacteria, peptidoglycan is heavily modified with carbohydrate-based anionic polymers such as lipo-teichoic acids (LTAs) and wall teichoic acids (WTAs) (Swoboda et al., 2010). Conversely, in Gram-negative bacteria, the peptidoglycan remains unmodified, as the modifications take place at the outer membrane level, characterized by the presence of lipopolysaccharides (LPS). The modifications occurring at the peptidoglycan level can potentially account for the variation observed in endolysins. This can help explain why endolysins of phages infecting Gram-negative bacteria tend to be single-domain globular proteins, with an individual enzymatically active domain (EAD) (Ghose and Euler, 2020; Kashani et al., 2017; São-José, 2018), while those endolysins of phages infecting Gram-positive bacteria present a modular structure being composed of an EAD, mostly located at the N-terminus, and a cell wall binding domain (CBD) at the C-terminus (Kashani et al., 2017; Rahman et al., 2021; São-José, 2018). By targeting the glycan or peptide components of peptidoglycan, as well as specific elements of (lipo)teichoic acids, CBDs enhance the proximity between EAD and its substrate (Loessner, 2005). Therefore, the catalytic activity and the specific cell wall ligand recognition are physically separated by a linker region. Based on this, the CBD is responsible for the high specificity, but it is not the only contributor to the endolysin spectrum of activity. Other factors, such as the ability of the EAD to cleave specific peptidoglycan bonds is of outmost importance to the spectrum of action (Ghose and Euler, 2020). For example, one of the EADs of the well-known LysK (endolysin derived from phage K, a broad-spectrum staphylococcal myovirus) appears to specifically cleave between the D-Alanine of the stem peptide and glycine of the pentaglycine cross-bridge (Fig. 3 A) (Becker et al., 2009a; Sanz-Gaitero et al., 2014). Consequently, peptidoglycan structures lacking this specific bond will not be degraded by LysK, even if the CBD is able to recognize and join to certain patterns in the cell wall structure. Another exclusive trait of some Gram-positive endolysins is the presence of multiple EADs and/or CBDs in different positions. For example, LysK consist of two different EADs and one CBD (Fig. 3 B) (Kashani et al., 2017). However, it has been reported in multiple studies that dual-EAD endolysins exhibit varying levels of catalytic activity. It is generally observed that one EAD plays a major role in the catalytic process, while the other EAD's contribution is

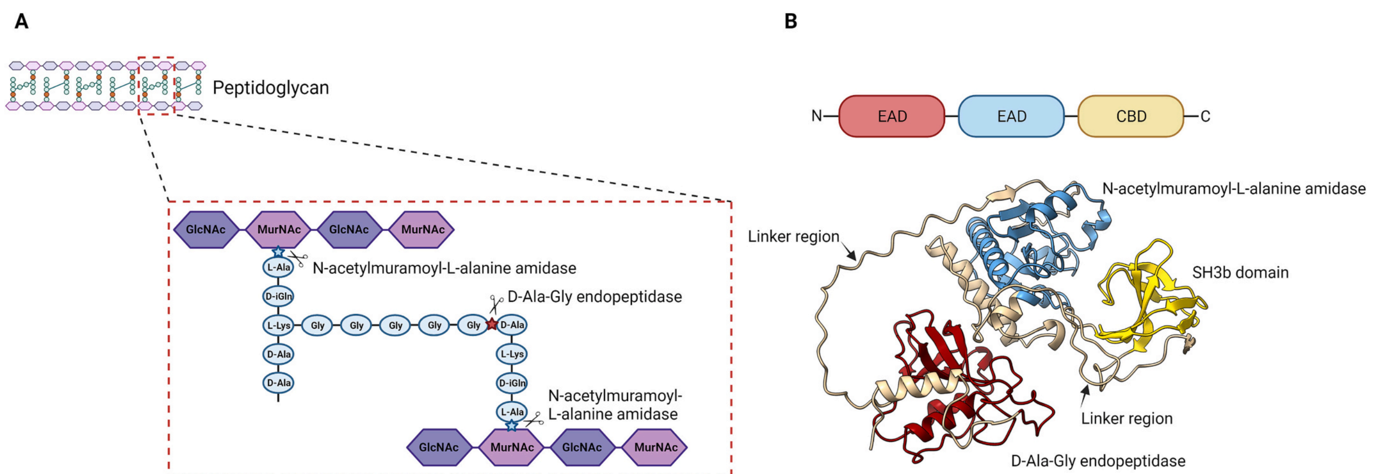

Fig. 3. A) Schematic representation of *S. aureus* peptidoglycan architecture, including LysK cleavage sites. In *S. aureus*, the stem is composed of the pentapeptide l-alanine-d-isoglutamine-L-lysine-d-alanine-d-alanine, and the bridge is a pentaglycyl segment. B) Predicted 3-D structure model of the endolysin LysK from *S. aureus* bacteriophage K, generated using AlphaFold2 (pLDDT = 85 pTM = 0.511). The structure consists of an N-terminal cysteine-histidine dependent amido-hydrolase/peptidase domain ($CHAP_K$) (red), a central amidase domain (blue), and a C-terminal SH3b cell wall-binding domain (yellow). $CHAP_K$ cleaves bacterial peptidoglycan between the tetrapeptide stem and the pentaglycine bridge. Enzymatically active domain (EAD) and cell wall-binding domain (CBD). The model aims to visually represent protein structure, rather than providing an exact depiction of the actual protein conformation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

minimal or even non-existent (Becker et al., 2009b; Donovan and Foster-Frey, 2008; Horgan et al., 2009). For that reason, it has been suggested that in some cases of dual-EAD endolysins, the central EAD do not play a role in linkage cleavage but in substrate binding, functioning as an auxiliary domain to the CBD (Son et al., 2018). However, it's important to note that there are exceptions to this trend (Nelson et al., 2006). Conversely, the requirement of a CBD has been questioned, as certain studies have demonstrated that the lytic activity remained unaffected or even improved upon CBD removal (Horgan et al., 2009; Mayer et al., 2012; Schmelcher et al., 2012). However, other studies showed the opposite result, where de presence of a CBD was essential for maintaining the lytic activity of the endolysin (Sass and Bierbaum, 2007; Zhang et al., 2019).

Different hypothesis can be proposed to explain why multidomain enzymes have evolved as the primary structures in phage endolysins that infect Gram-positive bacteria. One possible explanation may be that the peptidoglycan layer of Gram-positive bacteria consists of repetitive layers (up to 40 layers) (Gutiérrez and Briers, 2021), therefore such a thick barrier requires either the action of high amounts of endolysin molecules or the combined action of different EADs acting synergically to fully degrade the peptidoglycan barrier. Additionally, when multiple catalytic activities are combined in a single protein, it may lower the likelihood of peptidoglycan mutants that are resistant to treatment (Rodríguez-Rubio et al., 2013). Other hypothesis suggest that when bacteria are lysed, their internal contents, including endolysins, are released into the external environment. To avoid the potential harm to other bacterial hosts, multidomain endolysins may be disjointed by proteolysis with the consequent loss of activity (Oechslin et al., 2021). Other studies have proposed that after cell lysis, the CBD would maintain the multidomain endolysin tightly bound to the peptidoglycan debris, preventing their action on other bacteria (Loessner et al., 2002).

1.3. Endolysin cleavage site specificity

The peptidoglycan or murein polymer consists of a glycan strand that is made up of repeating units of the disaccharide *N*-acetylglucosamine (NAG)/*N*-acetylmuramic acid (NAM) linked by glycosidic bonds. These glycan strands are cross-linked through a peptide portion known as the stem peptide, which commonly consists of L-Ala-γ-D-iGlu (or γ-D-iGln)-*meso*-A_2pm (or L-Lys)-D-Ala-D-Ala (A_2pm, 2,6-diaminopimelic acid). This stem peptide is linked to NAM via an amide bond and connected to other stem peptide through a cross-linkage. In this context, the peptide moiety determines the different types of peptidoglycans among bacteria. For example, the presence of D-Isoglutamate (D-iGlu) and *meso*-A_2pm at position 2 and 3 of the stem peptide are typically found in most Gram-negative bacteria, on the other hand, D-Isoglutamine (D-iGln) and L-Lys replace these amino acids at the same positions in most Gram-positive bacteria (Vollmer et al., 2008). Regarding the cross-linkage between stem peptides, it can occur either directly via single interpeptide bond (most Gram-negative bacteria, such as *E. coli* (Fig. 4 A)), or through an interpeptide bridge (most Gram-positive bacteria (Fig. 4 B)). The interpeptide bridge is composed of a variable and diverse number of amino acids. Examples of interpeptide bridges in Gram-positive bacteria include $(Gly)_5$ in *Staphylococcus aureus* (Fig. 3 A), D-Asx (x can be either D-aspartic acid or D-asparagine) in *Enterococcus faecium*, L-Ala-L-Ala in *Enterococcus faecalis* and L-Ser-L-Ala in *Streptococcus pneumoniae* (São-José, 2018).

Based on the different types of linkages present in the peptidoglycan structure, endolysins can be classified according to their cleavage specificity as glycosidases, amidases, and endopeptidases (Danis-Wlodarczyk et al., 2021; Fenton et al., 2010; Latka et al., 2017) (Fig. 5). Glycosidases cleave the glycan portion of peptidoglycan and can be subdivided into muramidases (*N*-acetyl-*β*-D-muramidases), lytic transglycosylases and glucosaminidases (*N*-acetyl-β-D-glucosaminidases). Muramidases and lytic transglycosylases cleave the same glycosidic bond between NAM and NAG (Danis-Wlodarczyk et al., 2021; Ghose and Euler, 2020), but their catalytic mechanisms differ: hydrolytic for muramidases and non-hydrolytic for lytic transglycosylases (Kashani et al., 2017). On the other hand, glucosaminidases cleave the other glycosidic bond between NAG and NAM (Danis-Wlodarczyk et al., 2021). Amidases (*N*-acetylmuramoyl-L-alanine amidases) cleave the amide bond between the sugar (NAM) and the peptide moiety (L-Alanine, the first amino acid residue of the peptide stem). Finally,

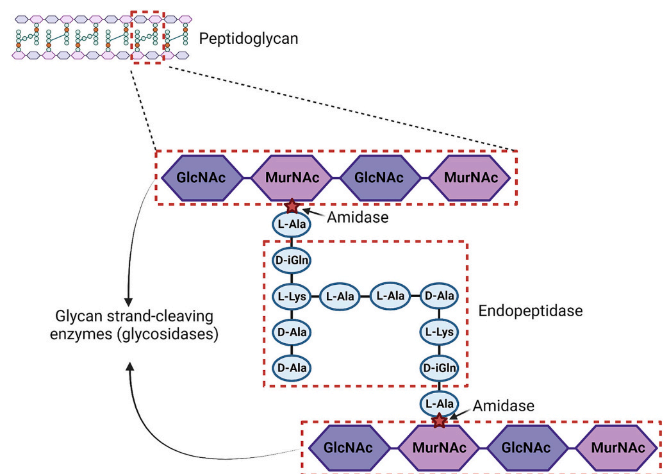

Fig. 5. Schematic representation of *E. faecalis* peptidoglycan structure, including the different endolysin cleavage sites.

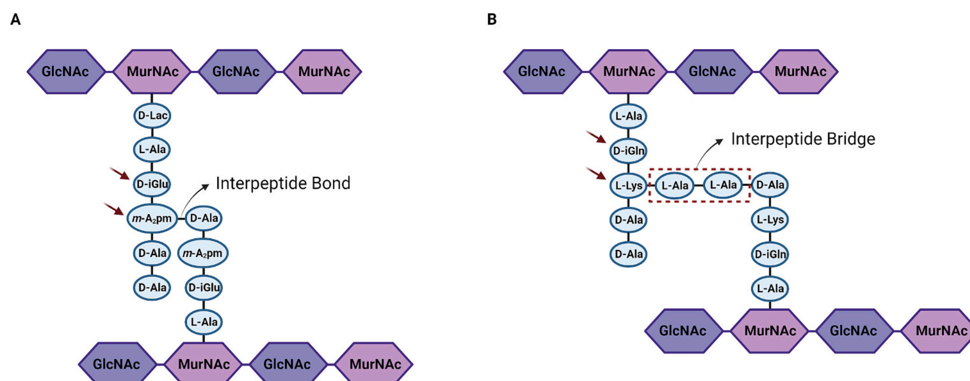

Fig. 4. Peptidoglycan architectures of *E. coli* (A) and *E. faecalis* (B). Principal differences are located at the stem peptide region (red arrows) and at the interpeptide connection among the different stem peptides, which can occur through single interpeptide bond (most Gram-negative bacteria), or via an interpeptide bridge (most Gram-positive bacteria). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

endopeptidases exhibit the most variability as they can cleave different peptide bonds within the stem peptides or the interpeptide bridges. Therefore, endopeptidases play a key role in defining the range of action of endolysins based on the presence of specific peptide linkages within a particular type of peptidoglycan.

2. Influence of endolysin's charged regions on gram-negative antimicrobial potential

As mentioned before, the presence of an outer membrane in Gram-negative bacteria is an obstacle to the proper action of most recombinantly produced endolysins. However, several studies have demonstrated that certain recombinant endolysins possess an intrinsic ability to cross the outer membrane with no need of other proteins. Based on these studies, the ability to pass through the bacterial outer membrane is facilitated by the presence of naturally occurring N-terminal (Plotka et al., 2020, 2019a; Wang et al., 2020a) or C-terminal (Chu et al., 2022; Vazquez et al., 2022; Vázquez et al., 2021b; Wang et al., 2020b) cationic peptides of different lengths. These positively charged regions appear to serve two functions. First, they engage in electrostatic interactions with the negatively charged molecules on the bacterial surface, thereby attracting the endolysin to the anionic outer membrane. Second, they enhance membrane permeability, aiding the endolysin penetration through the outer membrane and facilitating access to the peptidoglycan layer. Based on this, and considering the similarity of these properties to those observed in antimicrobial peptides (AMPs), it has been suggested that these regions would act as AMP-like subdomains within endolysins. Their presence at either the N- or C-terminus would be the evolutionary response to face the presence of these lipidic barriers in Gram-negative bacteria (Vázquez et al., 2021b). However, other studies have proposed the possibility that these AMP-like subdomains not only facilitate the enzymatic activity of endolysins but also play a role as a non-enzymatic mechanism to promote lysis by themselves (Vázquez et al., 2021a). In fact, it has been demonstrated that protein designs incorporating an AMP fused to a scaffold protein (GFP) can induce bacterial lysis, being this antimicrobial activity exclusively attributed to the presence of the AMP within the construct (Carratalá et al., 2020a; Carratalá et al., 2020b; Serna et al., 2017). Therefore, in some cases, the contribution of these AMP-like subdomains on the overall antimicrobial activity may be more relevant than that typically attributed to the enzymatic activity of the endolysin itself. Several studies have emphasized the critical importance of these positively charged regions as major contributors to the antimicrobial activity. For example, the bactericidal activity of endolysins LysC and Ts2631 was completely abolished when truncated variants lacking the highly cationic N-terminal extension were designed (Plotka et al., 2020, 2019b). Conversely, a synthetic peptide derived from the N-terminal region of LysC exhibited antimicrobial activity comparable to that of the wild-type enzyme (Plotka et al., 2020). Nonetheless, this feature is not restricted to the N-terminal region, as identical results were observed for the C-terminus. One of the first examples was described by Thandar et al. (2016) who demonstrated that the C-terminal amino acids 108 to 138 of phage endolysin PlyF307, named P307, were independently capable of effectively eliminating *Acinetobacter baumannii* (Thandar et al., 2016). In a more recent example, different punctual mutations on two key amino acids within the catalytic center of endolysin Pae87 had no effect in their bactericidal activity, suggesting that the AMP-like C-terminal region played a major role in its antimicrobial potential (Vazquez et al., 2022).

Notwithstanding the above, during the infective cycle of phages, Gram-negative endolysins containing an AMP-like region are thought to behave as canonical endolysins, being assisted by holins, spanins or disruptins (phage-encoded cationic antimicrobial peptides) (Holt et al., 2021) in order to completely disrupt the bacterial cell envelope. For that reason, it is unclear why some Gram-negative endolysins have evolved this positively charged terminal regions. Nonetheless, their identification has served for the rational design of recombinant endolysins with enhanced antibacterial properties when applied from outside.

3. De novo design

3.1. Increasing the net charge of endolysins

The previous section discussed how the presence of cationic extensions enhances the bactericidal effectiveness of endolysins by increasing their affinity for the bacterial surface and aiding in membrane permeabilization. However, when it comes to Gram-positive bacteria, which lack an outer membrane, a CBD is necessary for interaction with secondary cell wall polymer components or peptidoglycan itself. While CBDs are typically essential for EADs to access the peptidoglycan substrate, certain EADs show activity even in the absence of CBDs (Horgan et al., 2009). In fact, some of them exhibit superior activity profiles or a broader range of target hosts compared to the full-length endolysin (Mayer et al., 2011). In 2011, Low et al. suggested that the net positive charge of an EAD enables it to function independently of its CBD, presumably through ionic interactions with the negatively charged bacterial surface. In their study, Low et al. (2011) manipulated the activity of the catalytic domain of specific endolysins using structure-based mutagenesis. By modifying the electrostatic surface potential, they demonstrated that only engineered endolysins with a net positive charge displayed bacteriolytic activity (Low et al., 2011). Other studies reached similar conclusions when the net positive surface charge of other endolysins was increased, and consequently, their lytic activity was enhanced (Díez-Martínez et al., 2013). However, in 2019, Shang and Nelson, guided by the same engineering principles described by the findings of Low et al. (Low et al., 2011) and using the CHAP domain of PlyC endolysin, engineered a range of positively charged CHAPS mutants that did not display higher lytic activity than the wild type CHAP domain (Shang and Nelson, 2019). These contradictory results suggests that surface charge may not be the only mechanism that dictates the interactions between the endolysins and the bacterial cell wall. Nonetheless, in all of the aforementioned cases, endolysins were genetically modified through point mutations, which can result in unintended alterations beyond just modifying the overall charge, ultimately impacting the enzymatic activity. To address this issue, a recent study emphasized the relationship between surface charge of endolysins and the bacterial cell wall using two endolysins that only differed in their isoelectric points. The study established a positive correlation between the charge of the bacterial envelope and the lytic performance of peptidoglycan hydrolases (Wysocka et al., 2022).

3.2. Exploiting the potential of endolysin engineering

Endolysins, which have been extensively studied for their biotechnological applications, offer promising candidates for various engineering strategies. Notably, the literature provides examples of different modified forms, such as Lysocins, Innolysins, and Artilysins. **Lysocins** are bacteriocin-lysin hybrid molecules where the bacteriocin is responsible of surface receptor binding and periplasmic import through outer membrane protein channels (Lukacik et al., 2012; Yan et al., 2017). One example is PyS2-GN4 lysocin, that combines the *Pseudomonas aeruginosa* bacteriocin pyocin S2 and the GN4 endolysin, a muramidase from phage PAJU2 of *P. aeruginosa* (Heselpoth et al., 2019). On the other hand, **Innolysins** combine the enzymatic activity of endolysins with the binding capacity of phage receptor binding proteins (RBPs). Examples of this are Innolysins Ec21 and Cj1. Ec21 combines the monomeric Pb5 (RBP), located at the tail tip of the phage T5, with the LysEC8 endolysin (Zampara et al., 2020). Cj1 includes the tail fiber protein H of Campylobacter CAMSA2147 CJIE1-like prophage and the phage T5 endolysin (Zampara et al., 2021). These approaches are characterized by their narrow range of action since their mode of action is based on the specific recognition of certain receptors on bacterial surface, making them ideal as therapeutics for targeting specific bacteria with minimal

effects on the normal microbiome.

As discussed earlier, there is a relationship between the presence of positively charged regions at the end of some endolysins and their ability to overcome the outer membrane in Gram-negative bacteria. In these cases, the peptidoglycan barrier is more easily accessed from outside, which can result in a greater bacteriolytic effect. This observation is the basis for the design of **Artilysins**, which are endolysins that have been artificially modified to include positively charged amino acids at one of their ends. Given the absence of established guidelines for making these specific modifications, we will rely on the existing literature to shed light on this matter.

3.2.1. Optimizing Artilysin design: To N-terminal modify, or C-terminal modify? That is the Artilysin design question

To begin with, there is no consensus regarding the placement of the positive charge, whether it should be at the N- or C-terminus. Most existing studies focus on a single location, but very few investigate both positions. Briers et al. (2014b) were one of the first to contribute on this matter. They studied two different endolysins (PVP-SE1gp146 and OBPgp279) fused to a polycationic nonapeptide (PCNP) (Table 1). These protein designs included an N-terminal and C- terminal PCNP-fused version for each endolysin. After evaluating the antimicrobial capacities of the different versions, it was concluded that the N-terminal versions had a slightly better antimicrobial performance than their C-terminal counterparts (Briers et al., 2014b). Some years later, in 2018, Wang et al. came to similar conclusions with a different design that included the JDlys endolysin and a cell penetrating peptide (CPP) (Table 2). However, in this case, the C-terminal versions completely lost their bactericidal effect instead of only being slightly affected (Wang et al., 2018). More recently, Chen et al. (2021) generated two modified constructs by fusing the CecA peptide octamer (KWKLFKKI, Table 1) at either the C-terminus or the N-terminus of LysAB2 endolysin, and contrary to the previous results, the C-terminal modification was superior to the N-terminal modification (Chen et al., 2021). Although in this case, the N-terminal fusion construct also improved the antibacterial efficacy of the native LysAB2 (Chen et al., 2021).

Up to this point, it seems that none of the aforementioned studies have provided definitive guidance on the most suitable location for introducing positive charge. Nonetheless, there is one consistent observation. As previously stated, endolysins of phages targeting Gram-negative bacteria typically exist as globular proteins with a single EAD. However, modular structures can also be found, albeit in smaller proportions. In most studies involving N- terminal modifications, the focus was on modular endolysins, which consisted of a C-terminal enzymatically active domain and an N-terminal peptidoglycan binding domain (Briers et al., 2014a, 2014b; Lim et al., 2022b; Yang et al., 2015). In these cases, the position of the positive charges was located as far as possible from the EAD. This architectural arrangement may be advantageous as the amino acid tail is less likely to interact with the three-dimensional structure of the EAD, diminishing the chances of malfunction or aggregation due to an improper conformation. On the other hand, studies that primarily focus on C-terminal modifications typically involve globular endolysins (Antonova et al., 2020; Chen et al., 2021; Ma et al., 2017), suggesting that this location is less likely to affect the original catalytic activity of the enzyme once these extra amino acids are biosynthetically added.

The above evidence provides a picture that may be useful for decision-making when modifying endolysins by peptide addition. Based on the previous studies, when working with modular endolysins, the positive charge in form of a cationic peptide should be located on the opposite side of the EAD. On the other hand, C-terminal modification seems to work best when dealing with globular endolysins. However, it is important to note that these observations should not be considered as absolute rules or "dogma" for endolysin design, as exceptions can be found in the literature. For instance, the globular endolysin ST01, which comprises a lysozyme-like domain, was modified by fusing cecropin A (CecA) at the N-terminus (Table 1). This modification resulted in improved lytic activity against various Gram-negative pathogens, including *A. baumannii* (Lim et al., 2022a). Another example is AbEndolysin, a modular endolysin consisting of an N-terminal *N*-acetylmuramidase and C-terminal peptidoglycan-binding domain, which was also fused with CecA at the N-terminus (Table 1), right beside the EAD. This fusion construct exhibited increased bactericidal activity by at least 2-8 fold against various multidrug-resistant (MDR) *A. baumannii* clinical isolates (Islam et al., 2022). Finally, Chen et al. (2021), who previously demonstrated the superiority of the C-terminal fusion of the globular LysAB2 endolysin with CecA over the N-terminal modification, attempted to extend this C-terminal engineering to other three globular endolysins: PlyAB1, PlyE146 and an unpublished endolysin 68. Among them, only the C-terminal engineered PlyAB1 showed higher antibacterial activity than the native PlyAB1 against *A. baumannii* (Chen et al., 2021). In conclusion, while certain design patterns are recurrent in the literature, it is crucial to avoid considering these observations as general guidelines. The literature also presents instances of successful modifications that deviate from these patterns, highlighting the necessity for individualized evaluation on a case-by-case basis.

3.2.2. Optimizing Artilysin design: Are linkers important?

One of the main objectives of introducing a linker is to achieve certain physical distance between domains in fusion proteins. These molecular spacers have been extensively demonstrated to affect protein properties such as expression level (Amet et al., 2009), solubility (Zhao et al., 2008), and bioactivity (Bai and Shen, 2006). Furthermore, in modular endolysins, the different domains are connected by flexible linker regions of variable length (Schmelcher et al., 2012), and their importance have been widely demonstrated (Eichenseher et al., 2022; Hermoso et al., 2003; Pohane et al., 2015; Romero et al., 2018; Schmelcher et al., 2011).

Therefore, when designing a fusion protein containing an endolysin domain and a cationic peptide, uncertainties emerge regarding whether the connection between these two modules should be made through a linker region. Based on the literature, most studies either include no linker or employ flexible linkers of different length (Table 1). Flexible linkers are usually used when the joined domains require a certain degree of movement or interaction, which is particularly relevant when the cationic peptide needs to interact with the outer membrane. A study comparing the effect of a rigid and a flexible linker on the antimicrobial activity supports this approach. In this study, three modified variants of the endolysin LysECD7 and an optimized fragment of the sheep myeloid antimicrobial peptide 29 (SMAP-29) were produced: LysECD7-SMAP, LysECD7-flex-SMAP and LysECD7-rigid-SMAP (Table 1). Initially, all SMAP-modified molecules showed identical and improved bactericidal activity across a pH gradient range. However, when different conditions were tested (PBS buffer solution or human serum), LysECD7-flex-SMAP and LysECD7-SMAP appeared to be the most effective, being LysECD7-SMAP, lacking additional linkers, the variant that showed the best activity (Antonova et al., 2020). Therefore, for this type of protein design, flexible linkers or the absence of linkers seem to be the optimal choices. In fact, when linkers are used, the majority of studies employ this type of molecular spacers in their protein fusion constructs (Table 1).

Nonetheless, what about the impact of length? Another study focused on this issue. Here, the SMAP29 peptide was fused to the N-terminal of the globular LysPA26 endolysin, different linkers were used as molecule spacers between these two differentiated modules, resulting in three variants: SMAP29-GSA-LysPA26 (AL-3AA), SMAP29-(GSA)3-LysPA26 (AL-9AA), and SMAP29-(GGGGS)3-LysPA26 (AL-15AA) (Table 1). Among them, only AL-3AA showed improved antibacterial activity when compared to the unmodified LysPA26. AL-9AA exhibited similar antibacterial activity to LysPA26, while the antibacterial activity of AL-15AA was notably reduced. Therefore, the incorporation of SMAP29 may significantly enhance the bactericidal activity of LysPA26. However, the effectiveness of this improvement is influenced by the

Table 1
List of Artilysins designed to target Gram-negative bacteria.

Artilysin	Peptide	Sequence	Linker	Endolysin	Activity	Peptide location	Target Strain	Reference
Art-085	SMAP-29	RGLRRLGRKIAHGVKKYGPTVLRIIRIAG	–	KZ144	Transglycosylase	N-terminal	*P. aeruginosa*	(Briers et al., 2014a)
LoGT-001	PCNP	KRKKRKKRK	–	OBPgp279	Muramidase	N-terminal	*P. aeruginosa*	(Briers et al., 2014b)
LoGT-023	PCNP	KRKKRKKRK	AGAGAGAGAGAGAGAGAS	OBPgp279	Muramidase	N-terminal	*P. aeruginosa*	(Briers et al., 2014b)
LoGT-002	MW1	GFFIPAVILPSIAFLIVP	–	OBPgp279	Muramidase	N-terminal	*P. aeruginosa*	(Briers et al., 2014b)
AL-3AA	SMAP-29	RGLRRLGRKIAHGVKKYGPTVLRIIRIAG	GSA	LysPA26	Muramidase	N-terminal	*P. aeruginosa*	(Wang et al., 2021)
AL-9AA	SMAP-29	RGLRRLGRKIAHGVKKYGPTVLRIIRIAG	$(GSA)_3$	LysPA26	Muramidase	N-terminal	*P. aeruginosa*	(Wang et al., 2021)
AL-15AA	SMAP-29	RGLRRLGRKIAHGVKKYGPTVLRIIRIAG	$(GGGGS)_3$	LysPA26	Muramidase	N-terminal	*P. aeruginosa*	(Wang et al., 2021)
Artilysin A	PCNP	KRKKRKKRK	GAGA	Endolysin A	–	N-terminal	*H. pylori*	(Xu et al., 2021)
Artilysin B	PCNP	KRKKRKKRK	GAGA	Endolysin B	–	N-terminal	*H. pylori*	(Xu et al., 2021)
CecA::ST01	Cecropin A (CecA)	KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK	–	ST01	Transglycosylase	N-terminal	*A. baumannii*	(Lim et al., 2022a)
eAbEndolysin	Cecropin A (CecA)	KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK	–	AbEndolysin	Muramidase	N-terminal	*A. baumannii*	(Islam et al., 2022)
eLysMK34	Cecropin A (CecA)	KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK	$(AG)_3$	LysMK34	Muramidase	N-terminal	*A. baumannii*	(Abdelkader et al., 2022)
PlyA	Cecropin A (1-8)	KWKLFKKI	$(GGGGS)_2$	OBPgp279	Muramidase	N-terminal	*A. baumannii / P. aeruginosa*	(Yang et al., 2015)
DS-PA90	DS4.3 (CPP)	RIMRILRILKLAR	–	PA90	Transglycosylase	N-terminal	*A. baumannii*	(Lim et al., 2022b)
LysAB2-KWK	Cecropin A (1-8)	KWKLFKKI	GGSGG	LysAB2	Muramidase	C-terminal	*A. baumannii*	(Chen et al., 2021)
5aa	–	KRKRK	–	Lysep3	Muramidase	C-terminal	*E. coli*	(Ma et al., 2017)
10aa	–	KRKRKRKRKR	–	Lysep3	Muramidase	C-terminal	*E. coli*	(Ma et al., 2017)
15aa	–	KRKRKRKRKRKRKRK	–	Lysep3	Muramidase	C-terminal	*E. coli*	(Ma et al., 2017)
Mix	–	KRKRKFFVAIIP	–	Lysep3	Muramidase	C-terminal	*E. coli*	(Ma et al., 2017)
LysECD7-SMAP	SMAP-29 (1–17, K2,7,13)	RKLRRLKRKIAHKVKKY	–	LysECD7	Endopeptidase	C-terminal	*A. baumannii*	(Antonova et al., 2020)
LysECD7-flex-SMAP	SMAP-29 (1–17, K2,7,13)	RKLRRLKRKIAHKVKKY	GSAGSAAGSGEF	LysECD7	Endopeptidase	C-terminal	*A. baumannii*	(Antonova et al., 2020)
LysECD7-rigid-SMAP	SMAP-29 (1–17, K2,7,13)	RKLRRLKRKIAHKVKKY	AEAAAKEAAAKEAAAKA	LysECD7	Endopeptidase	C-terminal	*A. baumannii*	(Antonova et al., 2020)

*PCNP (Polycationic nonapeptide).
*CPP (cell penetrating peptide).

Table 2
List of Artilysins designed to target Gram-positive bacteria.

Artilysin	Peptide	Sequence	Linker	Endolysin	Activity	Peptide location	Strain	Reference
Art-240	PCNP	KRKKRKKRK	–	λSa2lys	Glucosaminidase and Endopeptidase	C-terminal	*S. agalactiae*	(Rodríguez-Rubio et al., 2016)
L-PTD12	PTD12	RKKRRQRRR	–	Lysostaphin	Endopeptidase	C-terminal	*S. aureus*	(Becker et al., 2016)
CPP_{Tat}-JDlys	CPP_{Tat}	YGRKKRRQRRR	–	JDlys	Amidase	N-terminal	*S. aureus (MRSA)*	(Wang et al., 2018)
LST_TAT	TAT	GRKKRRQRRRPPQ	–	Lysostaphin	Endopeptidase	C-terminal	*S. aureus*	(Röhrig et al., 2020)

*PCNP (Polycationic nonapeptide).
*CPP (cell penetrating peptide).

length of the linker region. This impact was further analyzed through protein conformational changes, revealing that the longer the linker between SMAP29 and LysPA26, the greater the negative impact on the secondary structure and the lower the antibacterial effect (Wang et al., 2021).

It is relevant to mention that these previous studies involved the modification of globular endolysins, where the peptide region was in close proximity to the EAD. The ineffectiveness of these longer linkers in these instances could be attributed to inefficient reduction of the interference of these protein domains with each other. In other words, the additional flexibility provided by longer linkers increases the chances of interfering with the three-dimensional structure of the protein, altering and deforming it, and causing a loss of function. In line with this, an increased length of the linker may have a neutral or positive effect on antimicrobial activity if located as far as possible from the EAD, for example, near the peptidoglycan-binding domain in a modular endolysin. This is exemplified by LoGT-023, which comprises an 18 amino-acid linker (AGAGAGAGAGAGAGAGAS) between the N-terminal PCNP tag and OBPgp279. This fusion design represents the best optimization of LoGT-001 (Table 1). In this study, an increasing length of the flexible linker between the PCNP and OBPgp279 resulted in a slightly higher antibacterial effect against *P. aeruginosa* (Briers et al., 2014b).

In conclusion, linkers have different effect depending on the structure of the endolysin. If the cationic charge is located as far as possible from the EAD, for example, close to the peptidoglycan-binding domain in modular endolysins, increasing the length of the linker appears to be harmless or, in some cases, beneficial for antimicrobial activity. On the other hand, in globular endolysins, an increasing length of the linker has proven to be detrimental for the antimicrobial activity. In these cases, shorter flexible linkers or the absence of a linker seem to be the preferred option.

3.2.3. Optimizing Artilysin design: Cationic and hydrophobic amino acids in peptide composition

One of the main features of Artilysins is the presence of a positive charge in form of a cationic peptide in the final fusion design. It has been hypothesized that the presence of these positively charged regions acts as a mechanism to attract the modified endolysin to the anionic outer membrane and destabilize their integrity in order gain access to the peptidoglycan layer, thereby potentiating the bacteriolytic effect. Among the literature, the most frequently used peptides are exclusively composed of cationic amino acids (PCNP) and AMPs, such us cecropin A, SMAP-29 or shortened versions of them (Table 1). The initial interaction between peptides and membranes is believed to be predominantly driven by electrostatic forces. This occurs when the positive charge of the peptide interacts with the anionic molecules present on the bacterial surface. Notably, a higher overall positive charge has been found to positively correlate with increased membrane penetration (Lee et al., 2021b; Riahifard et al., 2018), potentially elucidating the efficacy of PCNP. However, other factors rather than the overall charge may be influencing permeation properties. For example, the specific nature of cationic amino acids (particularly arginine rather than lysine) (Schmidt et al., 2012; Yang et al., 2018), the degree of hydrophobicity, along with the positioning of these hydrophobic residues and the adoption of a secondary structure (Hadjicharalambous et al., 2022) has been found to enhance the peptide's penetrative properties. AMPs combine all these properties (positively charged nature, amphiphilicity, and hydrophobicity), suggesting that these molecules may contribute more to membrane penetration than exclusively cationic peptides. There are limited examples in the literature that compare these two options, however, several studies have made initial attempts to address this issue. One particular study explored the effects of incorporating hydrophobic amino acids. These investigations have demonstrated that increasing the quantity of hydrophobic amino acids can strengthen the bactericidal capacity of endolysins, suggesting that hydrophobicity alone may play a significant role in the lytic effect (Yan et al., 2019). However, other studies have shown that when peptides with different properties (polycationic, hydrophobic, or amphipathic) were fused to various endolysins, those composed exclusively of cationic amino acids, specifically PCNP, demonstrated significantly higher bactericidal activity compared to variants modified with hydrophobic or amphipathic peptides capable of adopting a secondary structure (Briers et al., 2014b). This suggests that among the various properties influencing membrane permeability, the cationic nature may have a more pronounced contribution, but not exclusively. In a different study, Lysep 3 was C-terminally fused to four different peptides, creating four different variants: Mix (KRKRKFFVAIIP), 5aa (KRKRK), 10aa (KRKRKRKRKR), and 15aa (KRKRKRKRKRKRKRK) (Table 1). Among them, Mix was the only one that included hydrophobic amino acids into its sequence. Interestingly, despite this, Mix exhibited superior bactericidal activity against *E. coli* compared to 5aa and 10aa. Importantly, the positive net charge of 10aa was twice as high as that of Mix (Ma et al., 2017). While the cationic nature may indeed play a principal role, it appears that the optimal combination of hydrophobicity and charge density could significantly influence the antimicrobial activity. Actually, recent publications have extensively evaluated a wide range of peptides, predominantly consisting of antimicrobial peptides (AMPs), but also including synthetic constructs such as PCNP. These studies highlighted that the most effective peptide-endolysin combinations consistently involved the incorporation of AMPs. One of these studies revealed that among the 38 evaluated peptides, the fusion designs that included cecropin-related peptides emerged as some of the most active variants (Gerstmans et al., 2020). Similarly, a separate study investigated diverse peptide-endolysin configurations, evaluating a total of 42 peptides. The outcomes were consistent with previous research, highlighting the predominance of AMPs as the most active candidates (Duyvejonck et al., 2021). Although other factors, such as the location of the peptide or the characteristics of the fused endolysin, may have an influence, the inclusion of antimicrobial peptides (AMPs) in Artilysin designs, as opposed to peptides with a single characteristic such as cationic or hydrophobic, demonstrates a high effectiveness in penetrating bacterial membranes and enhancing bactericidal activity. This is attributed to the optimal combination of desirable characteristics such as hydrophobicity, charge density, and amphipathicity.

3.3. Alternative design options

Although Artilysins were initially developed as an engineering mechanism to surpass the outer membrane in Gram-negative bacteria, the addition of these peptides at the end of endolysins endows these enzymes with other appealing characteristics that improve their efficacy. Environmental factors, such as the presence of salts and the pH of the medium, have been observed to influence the lytic activity of endolysins. Several reports have demonstrated that modifying endolysins with cationic peptides leads to enhanced bacteriolytic effects in the presence of high concentrations of salt ions (Lim et al., 2022b) and across a broader range of pH values (Antonova et al., 2020). Importantly, this phenomenon is not limited to Gram-negative targeting endolysins. Artilysation can also be a viable approach for improving the cell wall affinity of Gram-positive targeting endolysins under a wider range of environmental conditions, including pH and ionic strength. This was precisely the aim of Art-240, a fusion of the PCNP to the C-terminus of λSa2lys (Table 2). λSa2lys is a modular endolysin consisting of two EADs (glucosaminidase and endopeptidase domains) and a CBD. The rationally designed Art-240 outperformed the unmodified λSa2lys by exhibiting increased enzymatic activity across a broad range of pH and NaCl conditions, resulting in a higher overall bactericidal effect (Rodríguez-Rubio et al., 2016).

The modifications presented thus far pertain to the second generation of endolysins, which involve the application of protein engineering techniques to enhance the antibacterial and biochemical properties of endolysins. However, the protein engineering and biochemical modifications aimed at optimizing the performance of endolysins in clinical settings, including enhancing immune responses upon systemic administration, extending their limited half-life, preventing proteolysis at the infection site, and effectively targeting intracellular bacteria, form the basis of a third generation of endolysins (De Maesschalck et al., 2020). At this stage, artilysation becomes a promising tool for creating third generation endolysins capable of effectively combating intracellular infections, such as those caused by *S. aureus*. *S. aureus* has the ability to invade eukaryotic host cells, enabling it to persist and proliferate intracellularly (Rollin et al., 2017) (Fig. 6 A). In this scenario, *S. aureus* evades the immune response and escapes from the action of antibiotics since these antimicrobial agents have limited ability to enter eukaryotic cells. This may explain the high recurrence observed in infections caused by *S. aureus* (Huitema et al., 2021; Peyrusson et al., 2020).

Endolysins have been proven to be efficient antimicrobials agents against *S. aureus* (Gutiérrez et al., 2021; Kaur et al., 2020; Lee et al., 2021a; Li et al., 2021; Manoharadas et al., 2021; Son et al., 2021). However, endolysins lack the ability to penetrate eukaryotic cells, making their efficacy against intracellular bacteria negligible (Rios et al., 2016). Artilysation may endow endolysins with the ability to penetrate eukaryotic cells, and this may be achieved through the fusion with cell-penetrating peptides (CPPs) (Borysowski and Górski, 2010) (Fig. 6 B). These short peptides have demonstrated the capacity to penetrate biological membranes and increase the internalization of different bioactive cargo molecules into eukaryotic cells (di Pisa et al., 2015; Järver and Langel, 2006). Therefore, fusion designs that combine *S. aureus*-targeting endolysins and CPPs may be a relevant strategy to combat these persistent forms of intracellular bacteria. A previous study addressed this issue, and while a triple-acting fusion protein combining LysK and lysostaphin (K-L) demonstrated the ability to invade cultured bovine mammary cells or murine osteoblasts, the intracellular killing efficacies were only moderate compared to the protein design incorporating a CPP (K-L-PTD1) (Becker et al., 2016). However, other recent studies have made more significant contributions in this context. For instance, in one of these studies JDlys was fused to three types of CPPs (CPP_{Tat}, CPP_{Ant}, CPP_{TP10}) at either the N- or C-terminus. The C- terminal fusions completely inactivated the enzyme, showing no lysing effect on *S. aureus*. As has been discussed in a previous section, this may be due to a possible conformational change induced by the fusion with the different CPPs. Among the N- terminal designs, only CPP_{Tat}-JDlys (Table 2) effectively eliminated intracellular methicillin-resistant *S. aureus* (MRSA), while CPP_{Ant}-JDlys, CPP_{TP10}-JDlys exhibited significantly reduced bactericidal effects compared to the unmodified JDlys (Wang et al., 2018). In a different study, an initial screening of 36 peptidoglycan hydrolases (PGHs) was conducted, from which seven were selected for subsequent modification. These selected PGHs were fused to six different CPPs, creating 42 combinations. Some of these designs were insoluble or insufficiently expressed and consequently were excluded from the experiment. The remaining designs showed either reduced or the same level of activity as the respective parental enzymes, being the Trans-activator of transcription (TAT) (Table 2) the one that had the least detrimental effect. The combination of the bacteriocin lysostaphin (LST) with TAT (LST-TAT) was the most

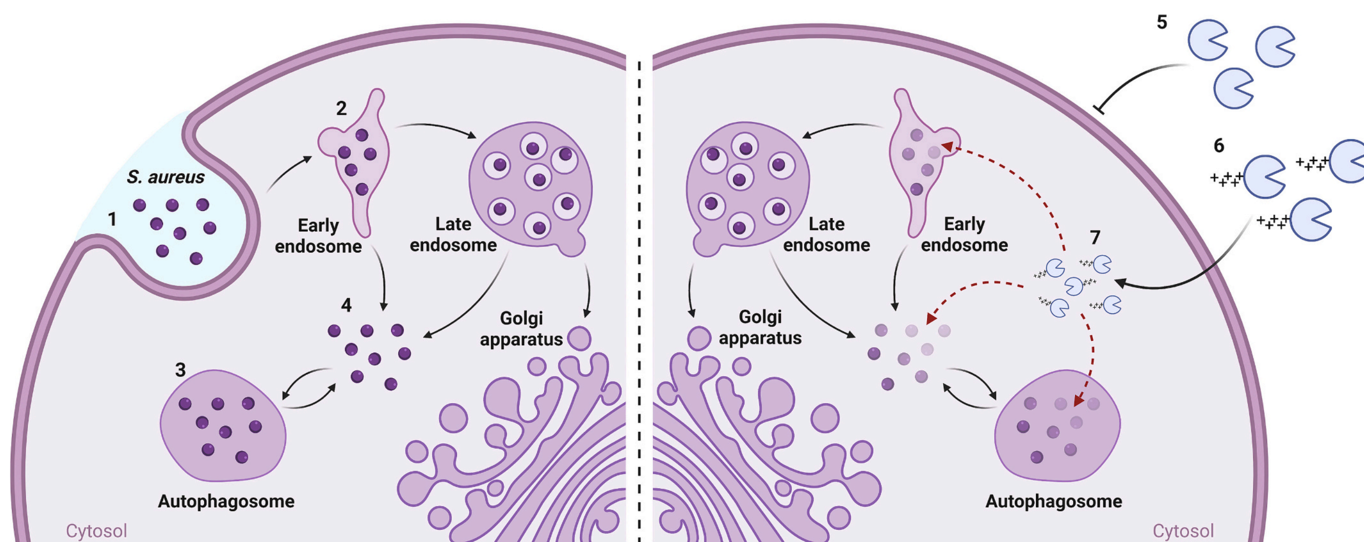

Fig. 6. Schematic overview of the intracellular preservation of *S. aureus* in eukaryotic cells. Left) *S. aureus* invades the cell and ends up inside the endosome (1). *S. aureus* may survive and grow within endosomes (2) or autophagosomes (3). *S. aureus* escape from endosomes into the cytosol, where it can persist inside the host cells, creating a bacterial reservoir protected from immune cells and antibiotics (4). Right) *S. aureus*-targeting endolysins are unable to penetrate eukaryotic cells (5). However, modification of endolysins with CPPs (6) allows these engineered proteins to transverse the eukaryotic membrane and target the intracellular *S. aureus*, eradicating these intracellular bacterial reservoirs.

effective construct, eradicating intracellular *S. aureus* in 8 out of 9 cases (Röhrig et al., 2020). A similar and more recent study came to the same conclusion, demonstrating the potential of the LST-TAT combination for targeting intracellular *S. aureus* (Keller et al., 2022). All this evidence suggests that, in addition to intracellular delivery, effective intracellular killing depends on other factors, such as identifying optimal combinations of highly active PGHs and compatible CPPs that do not impair the lytic activity of the PGH in a fusion construct.

3.4. Strategies and tools for maximizing artilysation design performance

Artilysation design is a multidimensional puzzle that demands profound knowledge, meticulous planning, and rational design. The preceding content of this review contributes to all these aspects, as it builds upon previous evidence and establishes guidance to improve the likelihood of generating successful protein designs. However, it is important to note that there are other methods that can assist in addressing these various aspects.

In the context of artilysation, databases play a crucial role in identifying candidates that possess the desired properties for incorporation into fusion protein designs. When it comes to peptides, there is a multitude of publicly available online databases that enable the searching and selection of specific peptides (Ramazi et al., 2022). One example is the Antimicrobial Activity and Structure of Peptides Database (DBAASP), which contains a vast collection of over 20,876 entries. This database offers comprehensive information about various aspects of peptides, including amino acid sequences, detailed structure, target species, target cell object, source, synthesis type, as well as the antimicrobial, hemolytic, and cytotoxic activities associated with each peptide (Pirtskhalava et al., 2021). On the other hand, the availability of dedicated databases that specifically focus on phage lytic proteins is limited. An example is PhaLP, a comprehensive database that integrates nine data types including proteins, phages, hosts, conserved domains, coding sequences, GO annotations, enzymatic activities, tertiary structures, and experimental evidence (Criel et al., 2021). By using these resources, researchers can rapidly identify the specific peptide and endolysin elements that possess the desired properties for fusing, thereby facilitating the development of a successful Artilysin design that aligns with their expected outcomes.

The present review offers insights into the design of Artilysins that are associated with higher success rates. However, it is not possible to establish a universal rule that is applicable to every type of design, as not all designs are composed of the same elements. The three-dimensional structure of proteins plays a vital role in their function, and merging two independent elements may result in clashes or disruptions that can hinder the overall functionality of the engineered protein. Furthermore, it is crucial to consider the potential impact of fusion on the stability and folding of the engineered protein, as these factors can significantly influence expression levels and may even result in misfolding or aggregation. For all these reasons, relying solely on one design may not be advisable, and evaluating different modular components and configurations of the same design is important to increase the chances of success. In essence, the phrase “putting all your eggs in the same basket” is not recommended when it comes to artilysation. Regarding this matter, Gerstmans et al. introduced the VersaTile technique in 2020. This method, specifically developed for modular endolysins, serves as a specialized and efficient DNA assembly technique to facilitate the rapid creation of engineered endolysins by integrating separate building blocks (peptides, linkers, and endolysins) referred to as tiles. In their study, Gerstmans et al. (2020) successfully generated a combinatorial library with a complexity of 9576 variants (calculated as $38 \times 2 \times 6 \times 21$) by combining all 38 peptides, 2 linkers, 6 CBDs, and 21 EADs tiles in a specific configuration: peptide-linker-CBD-EAD (Gerstmans et al., 2020). Taking it a step further, Duyvejonck et al. (2021) expanded on the previous achievement by creating five additional combinatorial endolysin libraries with different configurations, surpassing a total complexity of over 444,000 variants (Duyvejonck et al., 2021).

The combination of the aforementioned previous evidence described in this review, along with the potential offered by the VersaTile technique, presents an effective strategy for designing Artilysins with the desired properties. Based on the nature of the different building blocks, the information extracted from previous studies would guide the selection of the appropriate configuration and number of elements to combine, making the library to be created using the VersaTile technique more targeted and less complex. This is particularly significant since, despite the capability of one or several libraries to generate thousands of combinations, it is not feasible to evaluate all of them. Therefore, reducing their complexity through the application of a rational design based on previous evidence would be the most appropriate option (Fig. 7).

4. Conclusions

Endolysins are bacteriolytic enzymes that have emerged as promising candidates for the development of alternative antimicrobial agents. They specifically target and degrade the peptidoglycan layer of bacterial cell walls, leading to cell lysis and death. However, their use is limited by their inability to effectively penetrate the outer membrane of Gram-negative bacteria. The use of peptide-modified endolysins presents an exciting avenue for the biotechnological modification of these enzymes for therapeutic purposes. Artilysins, in particular, have been designed to overcome the outer membrane in Gram-negative bacteria by artificially introducing positively charged amino acids to their ends. However, there is no consensus on whether these modifications should be made. Evidence suggests that the location of the positive charge in endolysins depends on their architecture, with modular endolysins showing better results when the charge is located on the opposite side of the enzymatically active domain and globular endolysins showing better results with C-terminal modification. Linkers are important molecular spacers that affect protein properties, flexible linkers, or no linkers at all seem to be the best options, with linker length playing a crucial role in the antimicrobial activity of the protein. Artilysins are characterized by the presence of a positive charge in the form of a cationic peptide in their final design, facilitating interaction with the bacterial membrane. The optimal combination of characteristics such as positive charge, hydrophobicity, and amphiphilicity demonstrates greater effectiveness in penetrating bacterial membranes and enhancing bactericidal activity. Therefore, the inclusion of AMPs in Artilysin designs can be considered more advantageous than using solely cationic or hydrophobic peptides. Also, the combination of highly active peptidoglycan hydrolases and compatible CPPs shows promise in eradicating intracellular *S. aureus*. Databases and the VersaTile technique aid in identifying candidates and rapidly creating engineered endolysins with desired properties. Overall, the development of Artilysins represents an exciting advancement in the field of antibacterial therapeutics. Further research and optimization could lead to more effective treatments for bacterial infections, but careful assessment of each endolysin on a case-by-case basis is necessary to determine the optimal modifications.

Supervisor statement

We (Dr. Arís and Dr. Garcia-Fruitós), cosupervisors of Dr. Carratalá, give our consent to Dr.

Carratalá to act as a corresponding author in the manuscript entitled “Design Strategies for.

Positively Charged Endolysins: Insights into Artilysin Development” and we assume the.

responsibility of any misconduct associated with the submission.

Declaration of Competing Interest

The authors declare that they have no known competing financial

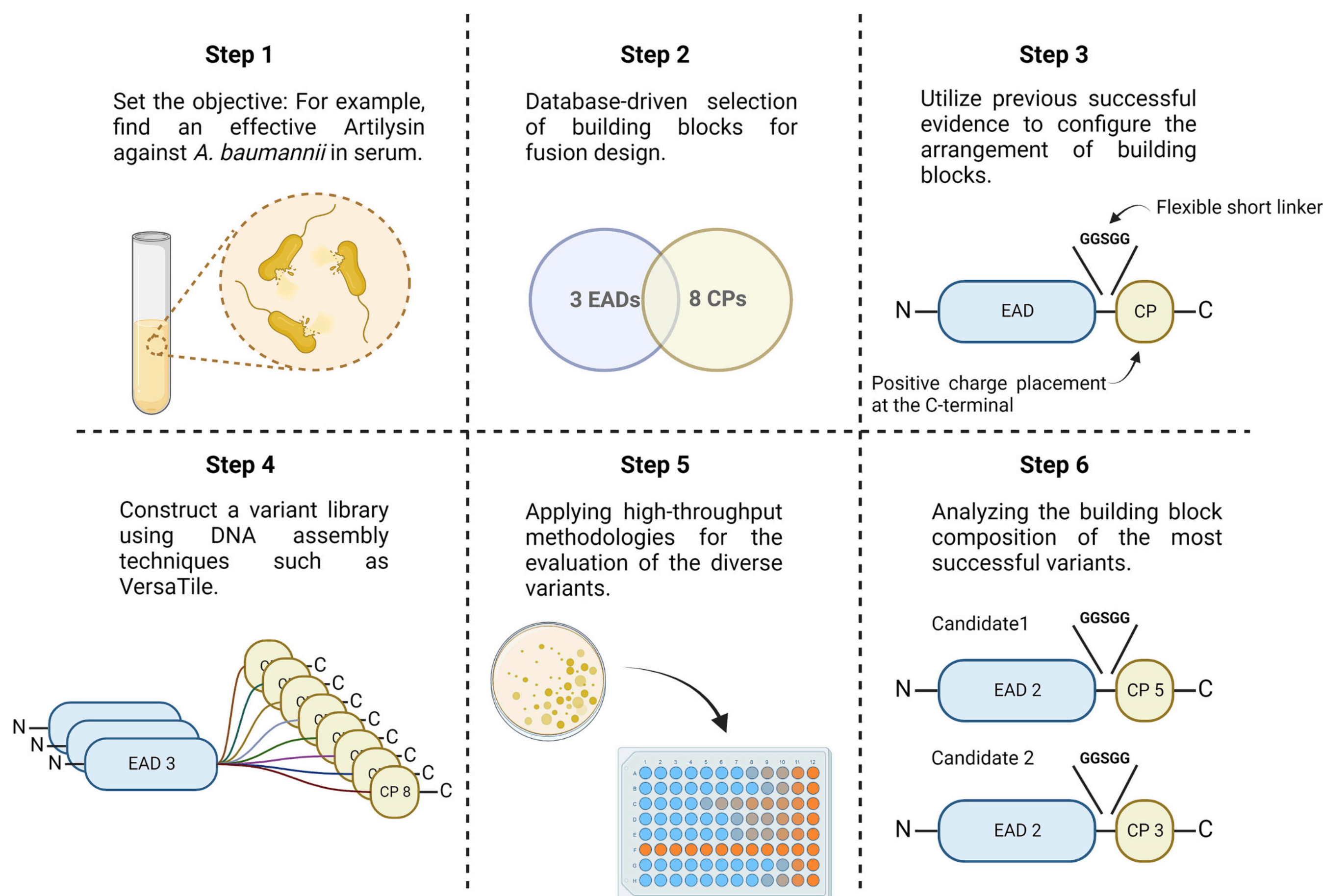

Fig. 7. Hypothetical workflow for effective Artilysin design. The various steps for Artilysin design are optimally summarized based on the guidelines described in the present work. It should be noted that the scheme is hypothetical and does not represent a real process. In order to simplify the schematization and facilitate understanding, the number of building blocks and configurations to be evaluated has been reduced. However, it is important to acknowledge that in practice, the complexity can be expanded, and a wider range of building blocks and configurations can be explored.

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