Peptidoglycan hydrolases as novel tool for anti-enterococcal therapy

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Enterococci are representatives of Gram-positive lactic acid bacteria. Most of them are constituents of commensal microflora on human and animal mucous membranes, but some strains have emerged as a major cause of nosocomial infections especially due to the presence of multi-drug resistance. It is anticipated that soon enterococcal infections will be untreatable by current antibiotics, and alternative means of combating infections caused by these organisms will be urgently needed. Finding new, more efficient therapeutics is a big challenge for pharmaceutical industry. One of the most potential novel alternative to classic antibiotics are peptidoglycan hydrolases; enzymes degrading the major component of bacterial cell wall. Thanks to their common features - bacteriolytic activity and narrow target spectrum, peptidoglycan hydrolases represent a safe tool for management of diseases caused by antibiotic-resistant enterococcal strains.

Keywords peptidoglycan; hydrolase; Enterococcus

1. Introduction

Peptidoglycan hydrolases represent a broad group of enzymes of different origin, but similar structure. Their substrate; peptidoglycan, is the most important component of bacterial cell walls. Activity of these enzymes results in a cell wall disruption and consequent bacterial cell lysis. They participate in many physiological processes in a bacterial life cycle; one class (endolysins) represent an important equipment of bacteriophage lytic arsenal, another is necessary in processes involved in a peptidoglycan reassembly (autolysins) or a competitive battle among related bacterial strains (bacteriocins).

Gram-positive bacteria are an appropriate target group for peptidoglycan hydrolases since in this case a peptidoglycan layer serves as the first defense line of cell integrity. Within this group of bacteria we can find many human and animal pathogens including some enterococcal strains. Infections caused by them become hard manageable due to the increasing antibiotic resistance and therefore it is important to find some novel and efficient antibacterial weapons. Peptidoglycan hydrolases seem to be a suitable alternative to antibiotics. An effectivity of some representatives was tested and demonstrated in vitro and in vivo in animal models. We can find a growing number of publications concerning characterization of these enzymes and their use in many different applications.

This paper is aimed at general characterization of peptidoglycan hydrolases, their features, classification, physiological properties, and potential applications. Last section of the paper presents findings obtained at our institute.

2. Bacterial cell wall

One of the most important elements of bacterial cell is a cell wall, which is responsible for cell shape maintenance and protecting against osmotic lysis. The strength and rigidity of the cell wall results from a layer of peptidoglycan, which is a covalent macromolecular structure consisting of strong glycan chains that are crosslinked by flexible peptide bridges [1] as shown in Fig. 1. The glycan strands are made up of alternating N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) residues linked by β-1→4 bonds. The D-lactoyl group of each NAM residue is substituted by a side peptide chain whose composition is most often L-Ala-D-Glu-L-Lys-D-Ala [2]. Neighbouring glycan strands are connected by crosslinking peptide chains between two side peptide chains. Crosslinking chains are the most variable components of peptidoglycan, and their composition is species-specific.

Peptidoglycan layer has some unique biophysical properties; on the one hand, it has the strength to withstand the cell’s turgor of up to 25 atmospheres, on the other hand it is not rigid, but flexible allowing reversible expansion of the cell wall [2].

2.1 Gram-positive versus Gram-negative bacteria

Bacteria can be divided into two basic groups; Gram-positive and Gram-negative. Formerly, this differentiation was based on the result of Gram staining, later it was uncovered that these different results were caused by diverse cell wall composition. Figure 2 shows general differences between these two types of cell walls. In the case of Gram-negative bacteria, there are three principal layers in the cell envelope; the inner (cytoplasmic) phospholipid membrane, the peptidoglycan cell wall, and the outer membrane made of lipopolysaccharides, phospholipids, lipoproteins and porins.

In Gram-positive bacteria the outer membrane is absent but the peptidoglycan layer in their cell wall is many times thicker than is found in Gram-negative bacteria [3]. Except peptidoglycan, Gram-positive cell wall contains one more important component, teichoic acids. These anionic polymers occur in two distinct forms depending on whether they...
are linked to the peptidoglycan (teichoic acid) or to the head groups of cytoplasmic membrane lipids (lipoteichoic acid) [4].

Fig. 1. Schematic representation of the peptidoglycan network structure. Long and strong glycan strands consisting of N-acetylg glucosamine and N-acetylmuramic acid subunits are crosslinked by short and flexible peptide bridges.

Fig. 2. Schematic structure of the cell envelope in a) Gram-negative, b) Gram-positive bacteria.

2.1.1 Enterococcal cell wall

Enterococcal species are common constituents of the intestinal flora of humans and animals. However, over the past few decades, some strains have emerged as frequent causes of multiple antibiotic resistant nosocomial infections [5]. As typical representatives of Gram-positive bacteria, enterococci have cell walls with very thick peptidoglycan layer close-fitting the cytoplasmic membrane. Walls of enterococci represent 27 to 38% of the dry cell weight and contain three main constituents; peptidoglycan, teichoic acid, polysaccharide, and sometimes, proteins are also mentioned. Variety and number of non-peptidoglycan polymers is related to bacterial species and growth conditions.

One of the characteristic traits of enterococcal cell wall is the structure of crosslinking peptide chain in peptidoglycan. The cross-bridge of most known Enterococcus species is formed by a single D-Asp residue [6].

3. Peptidoglycan hydrolases

Bacterial cell wall hydrolases represent various and numerous group of enzymes, which are able to cleave hydrolytically chemical bonds in peptidoglycan. Activity of these enzymes results in a cell wall disruption and consequent bacterial cell lysis. In the last years, they have been studied extensively in many laboratories all around the
world. The physiological functions of peptidoglycan hydrolases include the regulation of cell wall growth, the turnover of peptidoglycan during growth, the separation of daughter cells during cell division and autolysis [7]. They are also known to be produced by bacteriophages to digest the bacterial cell wall for phage progeny release at the end of bacteriophage lytic cycle [8].

3.1 Common classification
Sources of peptidoglycan hydrolases are very variable. They include animals, insects, plants, bacteria, and phages. Although enzymes from different sources may have similar structures and functions, they display important differences with significant impacts on their clinical applications [9]. According to their sources, peptidoglycan hydrolases can be classified into lysozymes, bacteriocins, autolysins, and endolysins.

3.1.1 Lysozymes
Lysozyme was discovered by Fleming in 1922. This bacteriolytic protein is phylogenetically ancient and almost ubiquitous among living organisms. It is probably the most studied enzyme in biology, biochemistry and medicine [10]. Lysozymes *sensu lato* are widespread in nature. They are produced by bacteriophages, bacteria, fungi, invertebrates, and vertebrates. In plants and animals (lysozymes *sensu stricto*), they represent a natural defense mechanism against bacterial pathogens [11]. The most known and commonly used representative of this group is hen egg-white lysozyme.

3.1.2 Bacteriocins
As early as a half century ago, a relatively broad study concerning bacteriocins was published. In this work, bacteriocins were characterized as a natural class of proteinaceous antibiotics, which in contrast to all other antibiotics, act only on strains of the same or closely related species [12]. Bacteriocin production has been detected in all major lineages of *Eubacteria* and in *Archaebacteria*. These highly specific enzymes are usually produced during stressful conditions and can eliminate neighbouring cells that are not resistant to their effect. The primary role of bacteriocins is probably to mediate intraspecific, or population-level, interactions [13]. Bacteriocins produced by Gram-positive lactic acid bacteria are predominantly small (< 10 kDa) proteins, but several large bacteriocins with cell wall hydrolytic activity have been described.

3.1.3 Autolysins
Autolysins represent a group of peptidoglycan hydrolases that digest the cell wall of bacteria that produce them. They are involved in numerous cellular physiological processes including cell growth, cell-wall turnover, peptidoglycan maturation, cell division, separation, differentiation, etc [14].

3.1.4 Endolysins
Bacteriophage endolysins are enzymes that are expressed near the end of the phage lytic cycle. Subsequent host cells lysis allows the bacteriophage progeny to escape from the host cell (“lysis from within”). The purified endolysins can also be exposed to bacteria externally, causing “lysis from without” [15].

3.2 Substrate specificity of peptidoglycan hydrolases
According to the specific bond in the peptidoglycan that is split down, peptidoglycan hydrolases can be divided into several groups, as shown in Fig. 3. Glycosidases (*N*-acetylglucosaminidases and *N*-acetylmuramidases) hydrolyze β-1→4 bonds between disaccharide units of glycan chains, peptidases are capable of cleaving short peptides (carboxypeptidases) or peptide cross-bridges (endopeptidases), and *N*-acetylmuramoyl-L-alanine amidases (amidases) cleave the amide bond between glycan chains and peptide bridges. Cleavage specificity depends on the catalytic domain of a peptidoglycan hydrolase [2, 16, 17] (see below).
3.3 Domain structure of peptidoglycan hydrolases

Molecule of typical peptidoglycan hydrolase consists of two domains, as shown in Fig. 4. With some exceptions, the N-terminal domain contains the catalytic activity (glycosidase, peptidase, or amidase) and is responsible for cleaving chemical bonds in the substrate – peptidoglycan. In some cases, two and perhaps even three different catalytic domains may occur in one enzyme molecule.

3.3.1 Binding receptors for CWB domains

C-terminal domain (called cell wall targeting – CWT, or cell wall binding – CWB domain) usually targets whole enzyme to the bacterial cell wall [8]. The activity level of peptidoglycan hydrolases does not arise only from catalytic domains. Efficient cleavage requires binding of CWB domain to its cell wall substrate. CWB domains do not recognize the peptidoglycan itself, since its structure is highly conserved. Correctly binding of whole enzyme molecule to the cell wall of sensitive bacteria requires certain specific receptors in peptidoglycan layer. This targeting ability offers the enzymes a high degree of specificity, since these receptors are only found in enzyme-sensitive bacteria [8, 19].

Receptors for some peptidoglycan hydrolases have been known for a long time, the nature of others is still being investigated. One of the formerly discovered is the receptor for CWB domain of pneumococcal autolysin (N-acetylmuramoyl-L-alanine amidase). Pneumococci have a nutritional requirement for choline and this amino alcohol seems to be incorporated exclusively into the teichoic acids of this bacterium. Replacement of choline in the growth medium with ethanolamine results in the formation of ethanolamine-containing cell walls, which were totally resistant against the action of the pneumococcal autolysin. Based on this finding, choline has been considered as a receptor for CWB domain of this enzyme [20].

Lysostaphin – bacteriocin from Staphylococcus simulans bv. staphyloyticus and its homologue, hydrolase ALE-1 from S. capitis specifically recognizes cell walls of S. aureus. These glycyglycine endopeptidases cleave the peptide cross-bridges in the peptidoglycan [21]. The CWB domain of these hydrolases has been classified as an SH3 domain and it has been uncovered that they recognize pentaglycine cross bridges in peptidoglycan, which are characteristic just for S. aureus [22].

On the other hand, there are some CWB domains with much wider range of receptors. For example, LysM (Lysin Motif) domain, which has been found in more than 4000 proteins of both prokaryotes and eukaryotes, represents the
main peptidoglycan-binding domain in bacteria. This domain binds to various types of peptidoglycan and most likely it recognizes the N-acetylglucosamine moiety [23].

3.4 Applications of peptidoglycan-degrading enzymes

Enzymes have been selected by natural evolution to favor the development of living organisms in their natural environment. Consequently, not all natural enzymes have adequate properties (specificity, stability, catalytic activity) to make them suitable for biotechnological applications, thus there are required several strategies to modify their natural properties [24].

Peptidoglycan-degrading enzymes have greatly been used in biotechnology industry to break cells. Major applications of these enzymes are related to the extraction of intracellular substances from bacterial cells and preparing spheroplasts for cell transformation. Other applications are based on the antimicrobial properties of these enzymes [17].

3.4.1 Obtaining intracellular products

Peptidoglycan hydrolases (principally lysozyme) are suitable tools for bacterial cell wall degradation in methods and techniques used for isolation of nucleic acids or releasing recombinant proteins from host bacterial cells. When intracellular products are obtained from Gram-positive bacteria, the action of lysozyme is sufficient [25]. In Gram-negative bacteria, the outer membrane prohibits access to lytic enzymes, thus in this case pre-treatment with a detergent or a cation chelating agent (as EDTA) is usually necessary to remove the outer membrane of Gram-negative cells [17].

3.4.2 Alternative to classic antibiotics

Enterococci have long been thought of as a harmless commensal of the mammalian gastrointestinal tract. However, in the last two decades they have become an important cause of nosocomial infections which are often difficult to treat due to the resistance to a large number of antibiotics, and an emerging resistance even to novel antibiotics used in the therapy is often reported in literature [26, 27].

One of the most potential novel alternatives to classic antibiotics are peptidoglycan hydrolases thank to their common features - bacteriolytic activity and narrow target spectrum. Several forms of these enzymes may be used in different applications, e.g. purified native enzymes, modified natural forms [28], recombinant enzymes prepared in a host organism and applied externally [29], or even overexpressed endogenously in transgenic plant or animal organisms to enhance their resistance against bacterial diseases [30].

The problem with infections caused by multidrug resistant bacteria also recalls the scientific interest in phage therapy, which has been relegated during the antibiotic era. Due to some potential risks in the application of whole phage particles (e.g. immunity response), purified phage endolysins seem to be a more suitable candidate.

3.4.3 Plant protection against phytopathogenic bacteria

The continuously increasing antibiotic resistance does not concern only human and animal pathogens but also phytopathogenic bacterial species. It is also clear that antibiotic resistance in plant bacterial pathogens can be transferred to humans via the food chain. In addition to the interest in using phage therapy (or purified phage endolysins) in human medicine, there is much interest in the application of phages as a biocontrol tool for bacterial species of agricultural importance [31].

3.4.4 Biocontrol of bacteria in food and feed

The empirical use of microorganisms and/or their natural products for the preservation of foods (biopreservation) has been a common practice for millennia. A fitting example is the group of lactic acid bacteria involved in fermentation of various kinds of food. A lot of studies have revealed that lactic acid bacteria produce an array of antimicrobial substances, including bacteriocins [32].

At present, the most commonly and commercially available bacteriocin is nisin but the bio-preserving effect of several more bacteriocins or a combination of nisin and lysozyme has been detected [32, 33]. Another promising way of food biopreservation again seems to be an application of bacteriophages or their endolysins, since they are capable to decontaminate raw products, such as fresh fruit and vegetables, to disinfect equipment and contact surfaces and to extend the shelf life of perishable manufactured foods [34].

3.4.5 Cosmetics and pharmaceutical products

Thanks to their antimicrobial properties, peptidoglycan hydrolases play an undeniably important role in production of cosmetic and pharmaceutical preparations. One of suitable candidates is again lysozyme, since its efficacy in therapy of infections of the skin and mucous membranes has been confirmed. Several years (or even decades) ago it was shown that lysozyme as an ingredient of ointments or poultices, accelerates the healing rate of burns and open skin wounds [35, 36]. At the last time bacteriophage endolysins were investigated as therapeutic agents for bacterial skin infections
in animals. Aerosolized PlyC endolysin was able to eradicate or significantly reduce pathogenic *Streptococcus equi* and was proposed as the first protein-based, narrow-spectrum disinfectant against this bacterial strain [37].

4. Known peptidoglycan hydrolases with anti-enterococcal activity

Enterococci are a leading cause of nosocomial infections, and treating these infections with conventional antibiotics has become increasingly difficult due to the acquisition of antibiotic resistance genes by these organisms [38]. Although several peptidoglycan hydrolases of diverse origin with activity against Gram-positive bacteria were identified and purified (e.g. Acd from *Clostridium difficile* [39], LytA from *Streptococcus pneumoniae* [40] or PL-1 from *Lactobacillus casei* phage [41] etc.), currently only two enzymes with anti-enterococcal activity are known - endolysin PlyV12 and enterolysin A.

4.1 Endolysin PlyV12

In 2004, PlyV12, a bacteriophage endolysin, was isolated and shown to effectively kill some enterococcal strains, as well as other human pathogens. PlyV12 is an amidase that exhibits a lytic effect on multiple *E. faecalis* (also vancomycin-resistant) strains. In contrast to other reported endolysins, PlyV12 was also found to be active against several pathogenic streptococcal and staphylococcal strains, making it one of the first endolysins with a spectrum of activity outside that of the host and closely related bacterial strains. This broad activity spectrum suggests the presence of a unique cell wall carbohydrate receptor common to these different human pathogens [38].

The molecule of PlyV12 consists of catalytic Amidase_5 domain and cell wall binding SH3_5 domain, as shown in Fig. 5.

Fig. 5. Schematic domain structure of endolysin PlyV12 obtained by the blastp analysis of its protein sequence using http://blast.ncbi.nlm.nih.gov/Blast.cgi.

4.2 Enterolysin A

Enterolysin A (EnlA), firstly described in 2003, is a heat-labile bacteriocin produced by *Enterococcus faecalis* LMG 2333 strain, with a broad inhibitory spectrum including selected enterococci, streptococci, pediococci, lactococci, and lactobacilli. Afterwards, an enterolysin A homologue was found to be produced by Gram-positive ruminal cocci and enterolysin A structural gene homologues were identified in several enterococcal and streptococcal bacterial species including *Streptococcus bovis* and *Enterococcus malodoratus* strains. Enterolysin A belongs to group of peptidoglycan hydrolases, with typical modular structure. The N-terminal region of the protein contains a domain found in the M37 family of metallopeptidases (catalytic domain). The C-terminal part of EnlA is distantly related to the known hydrolases only, but it shows significant sequence identity to two bacteriophage endolysins [42, 43]. This part has been thought to be responsible for enterolysin A binding to its cell wall substrate (putative CWB domain).

The heterologous expression of enterolysin A was studied in *Escherichia coli*. The expression of EnlA structural gene led to the synthesis and secretion of functional-active His-tagged enterolysin A protein (as shown in Fig. 6), which was purified to homogeneity and was shown to be fully active against the indicator strain. The expression of N-terminal or C-terminal part of EnlA and deletion of last 58 amino acids from C-terminal domain of EnlA led to the synthesis of biologically non-active proteins [44]. The activity of EnlA relies upon cell wall binding and C-terminal truncation of the protein completely abolishes its activity.

Fig. 6. Zone of growth inhibition caused by peptidoglycan hydrolase enterolysin A applied on sensitive *Enterococcus malodoratus* strain. Result of agar diffusion test.
In the following experiments, the putative function of enterolysin A C-terminal domain in cell wall targeting and binding was demonstrated. C-terminal domain was fused to GFP reporter and binding experiments using whole cells of various bacterial species as the target were performed. Figure 7 shows binding of fusion protein (named GFP-CWB) to bacterial cell walls, as visualized by fluorescence microscopy.

![Figure 7. Decoration of the surface of *E. malodoratus* NCDO846 after incubation with GFP-tagged C-terminal domain of EnlA hydrolase. Visualized by fluorescence microscopy.](image)

Data obtained using fluorescence binding assay correlated with sensitivity/resistance of bacterial species to EnlA hydrolase determined by agar diffusion test. As expected, GFP-CWB bound to the cells of bacteria classified as EnlA sensitive according to agar diffusion test, causing fluorescence of their surfaces, and did not bind to the surfaces of EnlA producing strain, or any bacterial species resistant to EnlA hydrolase. We demonstrated that the C-terminal region of enterolysin A is involved in specific recognition and binding to the target cell envelopes and represents true cell wall targeting domain.

5. Screening genomes of enterococci for genes encoding endolysins and endolysin-like proteins

Aside from enterolysin A, only three other bacteriolytic bacteriocins produced by Gram-positive lactic acid bacteria were described, namely zoozin A [45], stellalysin [46], and millericin B [47] and phages are probably the best source of anti-enterococcal endolysins. Although the number of phages infecting enterococci is sufficient, only few of them have been characterized at molecular level and the only known phage endolysin with activity against *Enterococcus* species is PlyV12. Much higher numbers of phage sequences were passively acquired as prophages in *Enterococcus* spp. genome sequencing projects. Therefore, we also analysed enterococcal genomes available in GenBank database for the prophage sequences. We found 62 protein sequences of endolysin-like enzymes. All obtained sequences were subsequently compared using BLASTP.

All of these endolysins appear to have a modular design (as described above). As shown in Table 1, these enzymes, with some exceptions, possess muramidase-like activity combined with LysM binding domain, or amidase-like activity combined with SH3_5 domain. Surprisingly, the Amidase_5 domain present in PlyV12 endolysin is rarely observed in endolysin-like proteins from enterococcal genomes, despite it is frequently encountered in endolysins from other members of *Lactobacillales* order (data not shown). The domain structure of bacteriophage endolysins represents a potential for construction of novel recombinant proteins with artificial combination of individual domains (so-called „domain shuffling”).

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Table 1. Basic characteristics of selected endolysin-like proteins from enterococcal genomes (data were obtained in march 2010)
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</tbody>
</table>

As a next step, a phylogenetic analysis of the catalytic and CWB domain sequences, respectively, using MEGA4 software [48] was performed (results not shown). The results obtained indicate that the degree of cognation among catalytic domains in the most cases corresponds in most cases with degree of cognation among CWB domains of individual proteins. One may hypothesize that at least in the case of peptidoglycan hydrolases the natural trend is distribution of complete genes (as well-tried domain combinations) rather than separate domains. Despite that, an artificial domain shuffling could be supposed as an indispensable method in enzymatic engineering.

### 6. Conclusions

Peptidoglycan hydrolases seem to be an interesting approach in the fight against multidrug resistant bacterial strains and in the future they could be an inestimable tool for treating diseases caused by these microorganisms. Purification of these proteins from their natural sources is often difficult and therefore preparation of recombinant hydrolases expressed in the new host organisms appears the more appropriate way to obtain them. Moreover, domain structure of peptidoglycan hydrolases offers some potential for enzyme engineering, since the combination of catalytic and CWB domains of different origin could lead in novel enzyme molecules with many desirable features. However, common use of these enzymes will require more studies and finding answers to many more research questions.

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### References


