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BACTERIOPHAGE ENCODED ENDOLYSINS AS POTENTIAL ANTIBACTERIALS

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Abstract:

Bacteriophages are viruses of the bacteria and are one of the most abundant life forms present on earth. They are extremely specific for their bacterial hosts even at the strain level. Their therapeutic potential was discovered in the beginning of twentieth century and was used in the countries of the former Soviet republic for treatment of common infections. However, with the advent of antibiotics in 1940s, the phage based therapy lost its momentum due to various limitations and primarily due to inadequate knowledge of phage biology. Antibiotics were like magic bullets for many severe infections for almost 70 years. However, the emergence of antibiotic-resistance in the past few years has posed a major threat to mankind and with the absence of newer antibiotics in the pipeline; alternative therapeutic strategies have to be adopted. Phage therapy is a promising alternative; however, due to many limitations phage encoded endolysins are potentially better candidates to antibiotics. Lytic phages during the final stages of their lytic cycle release enzymes called holins and endolysins that degrade the bacterial host cell wall and kill their hosts. These endolysins are very specific for their substrate cell wall components and if they are purified using recombinant DNA technology, can be used as potent antibacterials. In this review, we describe in detail the various uses of purified endolysins as antibacterials against Gram positive, Gram negative and mycobacterial hosts as well as their future improvements. Further modifications and protein-engineering approaches can enhance their antibacterial potential.

Keywords: Phage-therapy, Holin, Endolysins, Antibiotic resistance, Antibacterial

Introduction

Bacteriophages or simply phages are the viruses that specifically infect and replicate within their respective bacterial hosts. By their inherent nature they are also known as “bacteria eaters” and are estimated to be the most diverse and widely distributed and diverse entities in the biosphere [1]. Since, phages are natural predators of bacteria; they kill approximately 50% of the bacteria produced per day [2] . Phages were discovered in the pre-antibiotic time by Felix d'Herelle in 1917 who also realized their antibacterial potential [3].

There are two pathways through which phages infect bacteria. One is lytic cycle and other is lysogenic cycle. In case of lytic cycle, the lytic phages attach to bacteria, release their genetic material into the host cell where it utilizes the host cell machinery

to replicate and assemble into new progeny phages. Newly formed phages lyse their host and released outside to infect newer host cells [4]. In contrast, the lysogenic cycle does not result in host cell lysis, rather after the phage genome entry, it integrates with the host genomic DNA and replicates along with it harmlessly, or might even become established as a plasmid [5]. Lytic phages are generally considered more suitable for phage therapy as they lyse and kill their bacterial hosts but have several limitations, which will be discussed in detail later.

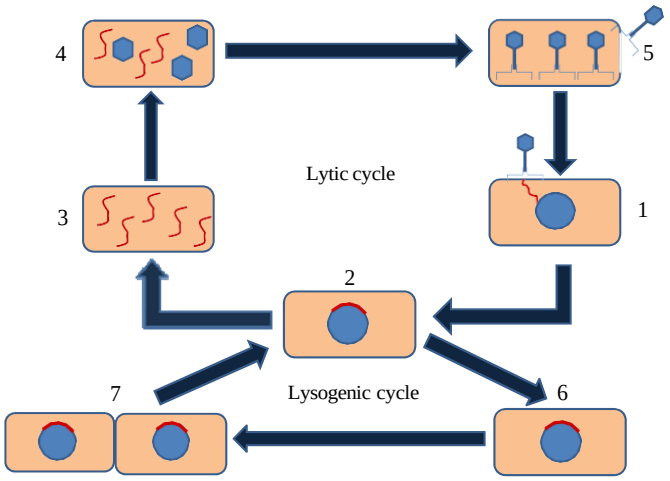

Fig. 1: Lytic and lysogenic life cycle of bacteriophage. 1. Phage attachment and release of the genetic material 2. Integration of phage genetic material into that of the host 3. Replication of viral genome 4. Assembly of viral components 5. Host lysis and the release of phage progeny 6. Phage DNA integrates itself with the bacterial genome and replicates. 7. Host cell divides and phage DNA passed onto the daughter cells.

The strategy for progeny release by bacteriophages following phage replication and dispersion enable phages to infect newer hosts. Few known filamentous phages, which continuously release their progeny across the cell wall without killing their host, but in case of non-filamentous phages, they lyse and exit their bacterial hosts by destroying the structural integrity of the peptidoglycan (PG) layer of bacterial cell wall [6]. For this purpose single-stranded RNA and DNA phages perform lysis of host by producing a single lysis protein without muralytic activity. In contrast, the double stranded DNA phages produce two proteins for host cell-lysis; the holins and the endolysins. Endolysins or lysins are muralytic enzymes known to degrade the cell wall while holins are membrane proteins. Lysins usually lack signal sequences and so are dependent upon holins to reach their substrate. During the final stages of the lytic-

cycle, the holins oligomerize and make channels into the plasma membrane to provide the passage for the endolysin to release and degrade the cell wall components. Lysin hydrolyse the peptidoglycan layer in the bacterial cell wall thus releasing mature phage progeny [6-7]. So the holin-endolysin system also known as the lambda paradigm, is universal to all dsDNA phages, with few exceptions [7].

Although phage therapy has been effectively used in former Soviet republican countries for treatment of various diseases but it suffers from its own set of demerits like; incomplete knowledge about the biology of the phages to be used in therapy, contamination of bacterial toxins during therapeutic phage preparations, inadequate knowledge of the phage receptors, lacking support from studies on animal models, frequent reversion to lysogeny and the most important drawback is the ethical issues for the use of live phages on human subjects. Hence, the use of phage-encoded endolysins which can be adequately purified and characterized seems to be a promising alternative to phage therapy[8].

Structure of Endolysin

General structure of lysins consists of two domains separated by a short linker region, the N-terminal catalytic domain (CD) which catalyses the hydrolysis of peptidoglycan and the C-terminal cell wall binding domain (CBD) that binds with the substrate [9]. Although endolysins share similar biological function, i.e. lysis of the infected host cell, endolysins represent a class of enzymes having large structural diversity. There are five types of catalytic domains in lysins which are identified, i.e. *N*-acetylmuramidases, *N*-acetylglucosaminidases, *N*-acetylmuramoyl-L-alanine amidase, *γ-D*-glutaminyl-*L*-lysine endopeptidase and endopeptidase, which specifically cleave the major bonds found in the peptidoglycan layer of host cell wall [10]. In contrast, the C-terminal domain or the cell wall binding domain binds to a specific substrate in the cell wall of the host bacterium. This binding is required by endolysin to carry out the cleavage of peptidoglycan bonds [11].

Fig. 2: Schematic representation of the structure of endolysin. CD stands for the catalytic domain; CBD is cell wall binding domain. CD and CBD are joined via the linker region.

Lysins as antimicrobial

It has been experimentally proved that endolysins show antimicrobial activity when applied externally. In the age of antibiotic resistance, phage endolysins have emerged as promising therapeutics against bacterial diseases due to their high

specificity and rare bacterial resistance. There is another term, "Enzybiotics" which was coined by Nelson et al in 2001 which refers to the approach of employing recombinant lysins to check bacterial infections [12].

Endolysins against Gram-positive pathogens

In general, Gram-positive cell wall is made up of a cytoplasmic membrane surrounded by a thick peptidoglycan layer where the peptidoglycan polymer chains are cross-linked by bacterial enzyme transpeptidase, which confers a rigid characteristic to their cell walls [13]. Apart from these, teichoic acids, lipids and a smaller volume of periplasm compose the Gram-positive cell wall. As the peptidoglycan layer is the outermost layer, it is easily accessible to endolysins when added exogenously. Endolysins cleave the specific bonds of the peptidoglycan layer upon contact, causing osmotic lysis and cell death eventually [14]. When applied externally, endolysins shows antibacterial activity against gram positive pathogens. For example, the endolysin LysH5 from bacteriophage ΦH5 against *Staphylococcus aureus* rapidly lyses bovine and human *S. aureus* , *S. epidermidis* strains in pasteurized milk [15].

Endolysins against Gram-negative pathogens

In case of Gram-negative bacteria, the cell wall consists of an inner cytoplasmic cell membrane surrounded by a comparatively thin peptidoglycan layer, and an outer membrane consisting of a lipopolysaccharide layer which is highly immunogenic [14].

When applied exogenously, endolysins have limited effect against Gram-negative bacteria, since their outer membrane consists of thick lipopolysaccharide layer that prevents access to the peptidoglycan layer [6]. So, to overcome this problem several engineered endolysins are being used. "Innolysins", are a type of such engineered endolysins that are constructed by combining endolysins with the Receptor Binding Proteins (RBPs). RBPs mediate adhesion specificity that form fibers or spikes at the phage tail while infecting bacteria. Thus, the RBP part allows the engineered enzymes to pass through the outer membrane while the endolysin part hydrolyses the peptidoglycan layer [16]. Another type of engineered endolysins are "Artilysins" that are made by fusing a LPS-destabilizing peptide with either the N- or the C-terminus of endolysins. This peptide possess amphipathic properties which interfere with the ionic and hydrophobic force of LPS layer causing its destabilisation, allowing the fused endolysin part to perform its function i.e. cleavage of peptidoglycan layer [17].

Endolysins against mycobacterial pathogens

The mycobacterial cell wall is structurally different from that of both Gram-positive and Gram-negative bacteria. Its cell wall consists of an inner plasma membrane surrounded by a thin peptidoglycan layer and an outer thick mycolate layer. The peptidoglycan and mycolate layers are held together by arabinogalactan, a type of polysaccharide [18]. This thick layer of mycolate and a layer of arabinogalactan raise hindrance for lysin function when applied externally. Therefore, phages against

mycobacteria which are known as mycobacteriophages synthesize two types of lysins; lysin A and lysin B [19]. Lysin A is a peptidoglycan hydrolase whereas lysin B is a mycolyl arabinogalactan esterase i.e. it cleaves the bond between mycolic acid and arabinogalctan [20]. Thus, on external application lysin B would cleave the bond between mycolate and arabinogalactan, giving access to lysin A to hydolyse the inner peptidoglycan layer.

It has been shown that lysin B from two different mycobacteriophages also exerts a bacteriostatic effect on *Mycobacterium smegmatis* in the presence of surfactants like tween80 [21].

Advantages and limitations of endolysins as antibacterial

Overcoming bacterial resistance: Bacteriophages are highly host specific i.e. they show genus, species or even strain specificity, which gives advantages over broad range antibiotics. Due to co-evolution of phages and their hosts, extreme specificity has resulted, leading to the cleavage of highly conserved and immutable targets present in the bacterial cell wall by endolysins. Thus, resistance has become a very rare event for the cell wall targets [7].

Synergistic effect: Synergistic effects produced by antimicrobial therapeutics can reduce its required doses and enhance the efficacy of treatment. For example, when pneumococcal phage lysins Cpl-1 and Pal and staphylococcal phage lysin LysK and lysostaphin are used against their respective pathogen, enhanced addictive effect was exhibited [22]. Also, when Cpl-1 was combined with antibiotics penicillin and gentamicin exhibited synergistic effects [23].

Safety & specificity: Due to high specificity of endolysins, these enzymes are specifically able to target the concerned pathogen without affecting the normal flora of the body. This is a major advantage over classical broad-spectrum antibiotics. But in case of systemic administration of endoysins, there is release of cellular debris like peptidoglycan segments; teichoic acid etc. caused by bacterial lysis which are proinflammatory in nature and may cause potential complications [24]. Hence, from the safety point of view, endolysin therapy still has some limitations which needs to be overcome.

Immunogenicity: As endolysins are proteinaceous in nature, they may trigger immune systems to produce antibodies. Taking different endolysins against different pathogens like *Streptococcus pyogenes*, *B. anthracis* and *S. aureus*, it was reported that antibodies against the lysins are raised but there was no adverse side effects or anaphylaxis in animal infection models and also the antibodies raised were not able to inactivate the enzyme[25]. An explanation for this phenomenon is that endolysin CBDs have high binding affinities and the fast kinetics of these enzymes helps to overcome the host's immune response [22b].

Effective against biofilms: Biofilm formation has serious medical implications, especially in catheters and other hospital associated infections, because biofilms can shelter pathogenic and multidrug-resistant bacteria. Biofilms have an outer Extracellular Polymeric Substances (EPS) coating within which microbes are protected [26] hence, they are more resistant or tolerant to antibiotics / disinfectants and more difficult to eradicate. Phages possess the ability to produce polysaccharide degrading enzymes, thus, they are capable of biofilm destruction by lysing the associated capsular EPS. It has been experimentally found that recombinant PlyGRCS obtained from the phage GRCS, is able to destroy *S. aureus* in a biofilm. PlyGRCS have one enzymatically-active domain which can cleave two types of bonds in peptidoglycan [27].

Concluding Remarks

We are living in an era where antibiotic resistance has emerged as a major concern. In this case, phage endolysins have come up as a long term alternative for antibiotics. Endolysins have potential to overcome major drawbacks like development of bacterial resistance. But still, there are concerns like accumulation of pathogen's cellular debris and immunogenicity leading to sepsis that are associated with using phage endolysins as antibacterials. Although this drawback is also associated with the use of antibiotics however, this has to be overcome in order to develop a successful antibacterial using endolysins. Techniques like protein-engineering, domain swapping etc. will help to enhance endolysin activity to many folds. Due to the modular nature of the endolysins, an endless number of chimeric proteins can also be engineered in order to create a lysin with an optimal level of activity and specificity against a group of pathogens. We anticipate successful use of endolysins as antibacterials or as "Enzybiotics" against different classes of sensitive as well as drug-resistant pathogens.

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