Gram-Negative Bacterial Lysins

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Contributor: Chad Euler

Antibiotics have had a profound impact on human society by enabling the eradication of otherwise deadly infections. Unfortunately, antibiotic use and overuse has led to the rapid spread of acquired antibiotic resistance, creating a major threat to public health. Novel therapeutic agents called bacteriophage endolysins (lysins) provide a solution to the worldwide epidemic of antibiotic resistance. Lysins are a class of enzymes produced by bacteriophages during the lytic cycle, which are capable of cleaving bonds in the bacterial cell wall, resulting in the death of the bacteria within seconds after contact. Through evolutionary selection of the phage progeny to be released and spread, these lysins target different critical components in the cell wall, making resistance to these molecules orders of magnitude less likely than conventional antibiotics. Such properties make lysins uniquely suitable for the treatment of multidrug resistant bacterial pathogens. Lysins, either naturally occurring or engineered, have the potential of being developed into fast-acting, narrow-spectrum, biofilm-disrupting antimicrobials that act synergistically with standard of care antibiotics.

Keywords: lysins ; endolysins ; bacteriophage ; ESKAPE ; Gram-negative ; Acinetobacter ; Pseudomonas ; E. coli ; Klebsiella

1. Introduction

The discovery of penicillin in 1928 at St. Mary's Hospital in London changed the course of infectious disease treatment and increased patient survival [1]. Antibiotics have been the cornerstone of healthcare [2]. Unfortunately, antibiotic use and overuse has led to the rapid spread of acquired antibiotic resistance [3][4]. Antimicrobial resistance (AMR) is a major threat to global health and human development, and is not merely a medical problem, but also an economic and social problem [5]. Each year in the U.S. alone at least two million people are infected with antibiotic-resistant bacteria, and at least 35,000 of these patients die [6]. Multidrug-resistant (MDR) Gram-negative infections are widely recognized as one of the greatest areas of unmet medical need, particularly carbapenem-resistant pathogens such as *Klebsiella pneumoniae*, *Acinetobacter baumanni*i, and *Pseudomonas aeruginosa*, which are on the rise among hospitalized patients [5][7][8]. It is predicted that by 2050 more than 10 million people will die per year due to AMR infections unless action is taken today to spur development of novel and nontraditional antibiotics [9].

2. Lysins for Gram-Negative Bacteria

Due to the structural barrier and the low permeability of the bacterial outer cell membrane, recombinantly expressed lysins have historically failed to gain access and cleave the underlying peptidoglycan to efficiently kill Gram-negative bacteria. The Gram-negative bacterial cell envelope is composed of protein, sugars and complex lipid arrangements [10]. Different approaches have been employed to increase the membrane permeability to lysins, such as the simultaneous addition of membrane destabilizing agents like poly-l-lysine, polymyxin B, or ethylene diamine tetraacetic acid disodium salt (EDTA), or modification of the lysins themselves, to include highly charged/hydrophobic aa residues [11][12][13][14][15] (Table 1). The success of these approaches has been varied, with few showing in vivo efficacy in animal models (Table 1) [16].

Table 1. Characteristics of Gram-negative Lysins.

Lysin	Predicted Enzymatic Activity	Antimicrobial Spectrum *	In Vivo Efficacy	Reference
LysAB2 and derivatives LysAB2 P3	**Lysozyme (Muramidase)**	***A. baumannii*, *S. aureus*, *E. coli***	**Bacteremia**	[17][18]
PlyF307 and derivatives P307SQ-8C	**Lysozyme (Muramidase)**	***A. baumannii*, *E. coli*, *K. pneumonia***	**Bacteremia, skin infection**	[16][19]

Lysin	Predicted Enzymatic Activity	Antimicrobial Spectrum *	In Vivo Efficacy	Reference
PlyE146	**Lysozyme (Muramidase)**	***E. coli*, *A. baumannii*, *P. aeruginosa*,**		[20]
LysABP-01	**Lysozyme (Muramidase)**	***A. baumannii*, *P. aeruginosa*, *E. coli***		[21]
PlyAB1	**Glycosidase**	***A. baumannii***		[22][23]
ABgp46	**Glucosaminidase**	***A. baumannii*, *P. aeruginosa*, *S. enterica* ser. Typhimurium**		[24]
Ply6A3	**Lysozyme (Muramidase)**	***A. baumannii*, *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *MRSA***	**Sepsis**	[25]
PlyPa103 and PlyPa91	**Lysozyme (Muramidase)**	***P. aeruginosa***	**Skin infection, pneumonia**	[26]
gp144 (KZ144)	**Transglycolase**	***P. aeruginosa*, *S. aureus*, *B. cereus***		[14]
EL188	**Transglycolase**	***P. aeruginosa***		[13]
LysPA26	**Lysozyme (Muramidase)**	***P. aeruginosa*, *A. baumannii*, *E. coli*, *K. pneumonia***		[11]
KP27	**Lysozyme (Muramidase)**	***K. pneumonia***		[27]
AP3gp15	**Lysozyme (Muramidase)**	***B. cepacia*, *E. coli*, *K. pneumonia*, *S. enterica* ser. Typhimurium**		[28]
EndoT5	**Lysozyme (Muramidase)**	***E. coli***		[15]
Lysep3 and derivatives	**Lysozyme (Muramidase)**	***E. coli*, *P. aeruginosa*, *Streptococcus sp.***	**Gastrointestinal infection**	[29][30]
Art-175 and Art-085	**Transglycolase**	***P. aeruginosa*, *A. baumannii*, *E. coli***	**Skin infection, sepsis**	[31][32][33] [34]
Lysocins	**Lysozyme (Muramidase)**	***P. aeruginosa***	**Bacteremia**	[35]
GN 121 and CF370	**Unknown**	***P. aeruginosa***	**Pneumonia**	[36]
Amurin APP2-M1	**Unknown**	***S. maltophilia***		[37]

* Organisms in bold depict the primary organism that the bactericidal activity of the lysin was tested against.

2.1. A. Baumannii Lysins

Carbapenem-resistant *Acinetobacter* causes at least 8500 infections per year in the USA, with an estimated 700 deaths in 2017 alone, and is considered to be an urgent threat by the Centers for Disease Control and Prevention [6]. *A. baumannii* has also received a Priority-1 classification by the World Health Organization for the development of new antibiotics, as few treatment options exist for infections caused by this MDR pathogen. Therefore, the development of new antimicrobials against *A. baumannii* are much needed, especially ones with novel mechanisms of action, like lysins. Multiple *A. baumannii*-specific lysins have been discovered, with a few showing the potential to be developed into a therapeutic agent.

LysAB2, an *A. baumannii*-specific lysin, was isolated from *A. baumannii* lytic phage ΦAB2 and has sequence homology to both glycoside hydrolases and to the muramidase/lysozyme-like superfamily [17]. Unlike Gram-positive lysins, Gram-negative lysins such as LysaAB2, are usually composed of an N-terminal lysozyme-like domain and a C-terminal positively charged region (Figure 1B). The C-terminal region of LysAB2 is composed of basic aa that are thought to bind to negatively-charged lipopolysaccharides (LPS) present on the bacterial outer membrane (OM), following which the N-terminal catalytic domain cleaves the bonds between the alternating N-acetylglucosamine and N-acetylmuramic acid of the bacterial peptidoglycan, leading to the lysis of the bacterium. LysAB2 has broad in vitro bactericidal activity towards both Gram-negative (MDR *A. baumannii* and *E. coli*) and Gram-positive (*S. aureus*) bacteria [17]. While the efficacy of LysAB2 in in vivo animal models of *A. baumannii* infection still remains to be elucidated, the bactericidal properties of

antimicrobial peptides (AMPs) extracted from the C-terminal of lysin LysAB2 have been tested in vitro and in vivo against MDR *A*. *baumannii* strains [17]. LysAB2 P3 was the peptide derivative that showed the highest level of antibacterial activity against *A*. *baumannii*, possibly due to alterations in the aa sequence that increased the net positive charge and decreased hydrophobicity of the peptide [18]. In an in vivo intraperitoneal (ip) infection model, LysAB2 P3 reduced *A*. *baumannii* CFU by 13-fold in ascites and 27-fold in blood and was able to rescue 60% of the bacteremic mice [18].

PlyF307, a naturally occurring 16kDa lysin, was identified from an environmental strain of *A. baumannii* using a broad expression-based screening approach [16]. In contrast to most two-domain Gram-positive lysins (Figure 1B), PlyF307 is composed of an individual muramidase enzymatic domain with a C-terminal, aa 108-138, that has a high positive net charge. Biochemical characterizations of PlyF307 and *A. baumannii* interactions have indicated that the target site of PlyF307 is located on both the OM and inner membrane (IM) of *A. baumannii*. In Gram-negative bacteria the outer leaflet of the OM is composed of polyanionic lipopolysaccharides (LPS) that are stabilized by divalent cations (Ca2+ and Mg2+). At higher pH and lower salt concentrations, the positively charged aa of PlyF307 are thought to establish ionic interactions with the OM and initiate the lytic process by providing the N-terminal enzymatic domain access to the underlying peptidoglycan, leading to the disruption of the IM and eventually, bacterial cell death [16].

PlyF307 has significant in vitro bactericidal activity against clinically relevant *A. baumannii* strains [16]. A 2 h incubation of 10^6 cfu/mL of *A. baumannii* with 100 μg/mL of PlyF307 resulted in a 5 $\log_{10}$ CFU/mL decrease in >80 clinical and environmental *A. baumannii* isolates. Additionally, while PlyF307 had no significant activity against *Pseudomonas* or *Staphylococcus*, a >2 $\log_{10}$ CFU/mL decrease was also observed against *E. coli* strains under the same conditions [16].

Due to the complex composition of bacterial biofilms, conventional antibiotics are generally unable to penetrate or degrade biofilms, or kill dormant bacteria harbored within the biofilms. As such, infections caused by biofilm-embedded bacteria like *A. baumannii* have become a major concern as they are more recalcitrant to treatment [38]. Luckily, a number of lysins have been shown to effectively destroy biofilms and kill the associated bacteria within these matrices [39]. The potency of PlyF307 has been demonstrated against *A. baumannii* biofilms formed on polyvinyl chloride (PVC) catheters [16]. A single, 2 h treatment with 250 μg of PlyF307 resulted in a >1.7 $\log_{10}$ CFU/mL decrease of the biofilm on the catheters. Additionally, observation of the catheters by scanning electron microscopy showed PlyF307 also destroyed much of the extracellular polymeric matrix of the biofilm. Furthermore, to simulate in situ treatment of indwelling catheters in patients, catheters with 2-day old biofilms were surgically implanted beneath the skin of mice. After 24 h, two doses of PlyF307 were injected subcutaneously to the site of the implant within a 4 h period. A 2 $\log_{10}$ CFU/mL reduction in *A. baumannii* viability under such experimental conditions suggests that it may be possible to treat infected implants with lysins as an alternative to surgically removing them.

PlyF307 is the first Gram-negative lysin to show in vivo efficacy as a therapeutic agent in a murine *A. baumannii* bacteremia model [16]. *A. baumannii* (10^8 cfu) were ip injected to allow the mice to develop systemic bloodstream infection within 2 h, at which time a single therapeutic dose of PlyF307 (1 mg/mouse) was delivered by ip injection. PlyF307 treatment saved 50% of the bacteremic mice, while nearly all of the control mice died within 24 h.

A similar phage–genome-based screening approach was used to identify PlyE146, encoded by an *E. coli* prophage. The recombinant lysin was shown to have N-acetylmuramidase activity against several Gram-negative bacteria, such as *E. coli*, *P. aeruginosa* and *A. baumannii* [20]. PlyE146 exhibited the greatest level of bactericidal activity against *A. baumannii*, resulting in >5 $\log_{10}$ CFU/mL decrease in 2 h compared to >3 $\log_{10}$ CFU/mL decrease against the *E. coli* strains tested. Similar to the abovementioned lysins, PlyE146 contains a highly cationic C-terminal domain that may play a central role in the observed potent bactericidal activity [16]. This activity may have also been aided by the presence of a 6-His aa tag, which increased the positively charge residues on the C-terminal. PlyE146 has a slower rate of killing than PlyF307, whereby the killing effects are observed after 30 min of exposure [16]. Additionally, PlyE146 activity is inhibited in the presence of serum, thus unlike PlyF307, it may have limited feasibility as a potential systemic treatment for *A. baumannii* [16]. The efficacy of PlyE146 still remains to be tested in in vivo animal models of *A. baumannii* infection.

Additional *A. baumannii* specific lysins have been identified and recombinantly expressed, such as LysABP-01, PlyAB1, ABgp46, and Ply6A3, which may be promising and may potentially be developed for the treatment of MDR infections of *A. baumannii* (Table 1) [16][21][23][25]. Lysin LysABP-01 isolated from *A. baumannii* lytic bacteriophage ABP-01 has bactericidal activity against *A. baumannii*, *E. coli* and *P. aeruginosa* [21]. LysABP-01 has synergy with various antibiotics such as colistin, an antibiotic of last resort that is used to treat MDR infections [21]. Another lysin, PlyAB1, was isolated from *A. baumannii* bacteriophage ABp1, has bactericidal activity against 48 hospital-derived pan drug-resistant *A. baumannii* isolates [22][23]. Lysin ABgp46 isolated from *A. baumannii* phage Øvb_AbaP_CEB1 has bactericidal activity

against several multidrug-resistant *A. baumannii* strains [24]. In the presence of membrane permeabilizing agents such as citric acid and malic acid, ABgp46 was active against additional Gram-negative pathogens such as *P. aeruginosa* and *Salmonella enterica* serovar Typhimurium. Lysin Ply6A3 produced by bacteriophage PD-6A3 was shown to kill 70% of MDR *A. baumannii* strains and protect 70% of mice in a lethal *A. baumannii* sepsis challenge model [25].

2.2. P. Aeruginosa Lysins

MDR *P. aeruginosa* nosocomial infections led to 32,600 cases and 2700 deaths in 2017 [6]. Such infections manifest as urinary tract and surgical site infections to more complicated pneumonia and bloodstream infections. Numerous strains of *P. aeruginosa* are resistant to many different classes of antibiotics, including the broadly used carbapenems [40]. Lysins that effectively kill *P. aeruginosa* could be useful in controlling the spread of *P. aeruginosa.*

Using an in silico approach to search *P. aeruginosa* phage genomes, the Fischetti laboratory has identified 16 *P. aeruginosa* specific lysins that had phylogenic similarities to *A. baumannii* lysin PlyF307 [16][26]. Of the 16 identified lysins, two lysins have shown promise as drug candidates: PlyPa03 and PlyPa91. Both lysins have displayed bactericidal properties against a number of clinical strains of *P. aeruginosa*, including biofilm-embedded strains. Unfortunately, both lysins were serum sensitive, thus limiting their use as a systemic therapeutic. However, PlyPa91 showed in vivo efficacy in a mouse model of *P. aeruginosa* skin infection, reducing bacterial CFUs by >2 $\log_{10}$s. PlyPa03 also had efficacy in a mouse model of *P. aeruginosa* pneumonia, where 70% of mice treated with two intratracheal and intranasal doses of PlyPa03 survived [35].

Additional lysins with antipseudomonal activity have been identified and tested mostly in in vitro bactericidal assays in the presence of OM permeabilizers (Table 1) [11][13][14]. Lysin gp144 (KZ144), isolated from the *P. aeruginosa* phage phiKZ, has one of the few Gram-negative modular lysins. It has an N-terminal peptidoglycan binding domain and a C-terminal catalytic domain with transglycosylase activity [14]. Gp144 was able to degrade chloroform-treated *P. aeruginosa* and, to a lesser extent, *S. aureus* and *B. cereus.* Lysin EL188, isolated from *P. aeruginosa* phage EL, has potent bactericidal activity in combination with OM permeabilizers such as EDTA [13]. A combination of EL188 and EDTA led to a >4 $\log_{10}$s reduction of *P. aeruginosa* in 30 min. While the potential use of EL188 was diminished by the need of co-administration of EDTA or similar chemicals, this combination may be suitable for use on local topical infections, such as those in burn patients [13]. LysPA26 was isolated from phage JD010. Based on sequence analysis, LysPA26 was shown to be part of a lysozyme-like domain family of superfamily cd00442 [11]. LysPA26 was capable of killing biofilm-embedded *P. aeruginosa* without the addition of outer-membrane permeabilizers and also has bactericidal activity against to *K. pneumoniae*, *A. baumannii* and *E. coli* [11]. To date, in vivo studies have not yet been performed to look at the therapeutic potential of these lysins.

2.3. Lysins Against Other Gram-Negative Pathogens

Additional recombinant bacteriophage lysins that have targeted other Gram-negative pathogens have shown mostly in vitro bactericidal activity, such as those against *K. pneumonia*, *E. coli*, *Burkholderia cenocepacia* and *S. enterica* ser. Typhimurium (Table 1). Lysin KP27, isolated from environmental phage vB_KpnM_KP27, has bactericidal activity against multidrug-resistant *K. pneumonia* [27][28]. AP3gp15, a muralytic enzyme originally encoded on the *Burkholderia* AP3 phage, has broad in vitro antibacterial activity against *B. cenocepacia*, as well as *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *S. enterica* ser. Typhimurium [27][28].

Several *E. coli* lysins have been identified and recombinantly expressed to date, such as the T5 lysin (EndoT5) and Lysep3 [15][29]. EndoT5, a peptidoglycan hydrolase belonging to the M15 family of peptidases, was able to lyse *E. coli* strains in vitro in the presence of various outer-membrane permeabilizers such as polymyxin B, gramicidin D, poly-l-lysine, chlorhexidine and miramistin [15]. Lysep3, a coliphage lysin isolated from the *E. coli*-specific bacteriophage vB_EcoM-ep3, has in vitro bactericidal activity against *E. coli* and *P. aeruginosa* [29]. Many modifications have been made to Lysep3 to increase its ability to penetrate the outer membrane. Lysep3 bactericidal activity was enhanced by fusing the *Bacillus amyloliquefaciens* bacteriophage lysin binding domain D8 to the C-terminal region of Lysep3 [30]. Additional variations include Lysep3 fusions with Colicin A, addition of positively charged aa to increase the number of positive charges and hydrophobic residues on the N or C-terminal domains, etc. [30]. The following section describes further methods used to modify lysins so as to enhance the therapeutic potential of these molecules to effectively kill Gram-negative bacteria.

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