ATHABASCA UNIVERSITY

LYSINS AS POTENTIAL ANTIBIOTICS: IDENTIFICATON OF CANDIDATE LYSINS WITH PROPOSED ANTIMICROBIAL PEPTIDE-LIKE PROPERTIES THAT TARGET *PSEUDOMONAS AERUGINOSA*

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JILLIAN NICKEL, BSC., NM(MRT)

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Submitted by

Jillian Nickel

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The thesis examination committee certifies that the thesis
and the oral examination is approved

Supervisors:
Dr. Steven Johnson
Athabasca University

Dr. Shawn Lewenza
Athabasca University

External Examiner:
Dr. Evan F. Haney
University of British Columbia

April 22, 2025

1 University Drive, Athabasca, AB, T9S 3A3 Canada
Toll-free (CAN/U.S.) 1.800.788.9041 ex. 6821
fgs@athabascau.ca | fgs.athabascau.ca | athabascau.ca

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Abstract

Introduction

The discovery of bacteriophages that have specific antimicrobial activity against bacteria was prior to the turn of the 20th century. More recently, viral components of bacteriophages, such as lysin, have been described for their lytic activity against antibiotic resistant bacteria. Given the widespread antibiotic resistance in the most common bacterial pathogens, this project aims to better understand how lysins kill bacteria and to identify new lysin candidates that may function as better antimicrobials. Due to an almost infinite number of bacteriophages on the planet, obtaining specific lysins for application in clinical research for use against antibiotic resistant pathogens requires further understanding of their structure and function. Lysins have demonstrated antimicrobial activity in the laboratory setting, and when applied in animal models of infection. Lysins are enzymes that are produced and assembled inside a host bacterium during phage infection. These enzymes typically degrade the bacterial cell wall, with access from the interior of the infected cell. We hypothesized that the C-terminus of lysins from Gram-negative phages will have more antimicrobial peptide properties than Gram-positive phage lysins, which should promote enhanced killing of Gram-negative bacteria. Lysins that can disrupt the outer membrane can gain access to the cell wall from both sides of the membrane.

Methods

Using the PhaLP database of predicted phage proteins, we recovered all lysin proteins from the model Gram-negative and Gram-positive pathogens, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, respectively. The total number of lysins was reduced by removing all duplicate lysin sequences, as identical lysins were common. Next, we examined the hydrophobicity, net charge, as well as the arginine to tryptophan frequency in the C-terminal 50 amino acids of all lysins, to determine if there was a difference between lysins from Gram-negative and Gram-positive bacteria.

Results

This analysis indicated that the C-terminal portion of lysins from *P. aeruginosa* contained more hydrophobic amino acids, higher net positive charge and a higher ratio of lysin/arginine amino acids when compared to *S. aureus* lysins. Secondary structure analysis of priority lysins had predicted alpha helical structure, which is characteristic of antimicrobial peptides.

Conclusion

By examining the C-terminal domain of phage lysins, we identified more features of antimicrobial peptides in lysins that originate in phages that kill Gram-negative bacteria. Two primary lysins were identified as the lead candidates, as well as six additional candidate lysins for future experimental research to test their antimicrobial efficiency. This work supports and extends the observations that lysins contain domains in their C-terminus that might function to disrupt the outer membrane of Gram-negative bacteria, thereby enhancing the antimicrobial activity of phage lysins. With this potentially new domain, lysins can access the cell wall from the interior or exterior of a Gram-negative bacterium, and therefore should result in stronger antimicrobial activity. This research has identified antimicrobial enzyme candidates to combat the inherently antibiotic resistant Gram-negative pathogen, *P. aeruginosa*.

Keywords: lysins, antimicrobial peptides, bioinformatics

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Literature Review

The World Health Organization (WHO) lists antimicrobial resistance as one of the top 10 threats to global health on their website and further declares that drug resistant diseases could cause 10 million deaths each year by 2050. It also points out that such increasing resistance to antibiotics make more common diseases such as sexually transmitted infections, urinary tract infections, and respiratory infections, increasingly more difficult to treat, putting significant increased strain on health care systems around the world according to the World Health Organization (World Health Organization, 2024). The World Bank estimates that these such infections could result in $1 trillion USD in additional health care costs by 2050, as well as GDP losses of $1 trillion to $3.4 trillion USD per year by 2030. If resistance continues across the bacterial species with the bacterial classes that are a threat to human health, there will be no means to fight these infections which could have exhaustive effects on our health care organizations world-wide as well as increased death-rates.

Priority antibiotic resistant bacteria pathogens

Antibiotic resistant infections are occurring with increased prevalence as well as complexity in terms of treatment approach with the currently available therapeutic options. The WHO established a list of antibiotic resistant bacteria according to their priority (critical, high and medium) for the specific aim of developing successful antibiotic treatments through continued research (Breijyeh et al., 2020). The species that fall under the critical category include: *Acinetobacter baumannii* (carbapenem-resistant), *Pseudomonas aeruginosa* (carbapenem-resistant), *Enterobacteriaceae* (carbapenem-resistant, ESBL-producing). And such species that fall under the "high category include: *Enterococcus faecium* (vancomycin-resistant),

Staphylococcus aureus (methicillin-resistant, vancomycin-intermediate and resistant) *Helicobacter pylori* (clarithromycin-resistant), *Campylobacter* spp., (fluoroquinolone-resistant), *Salmonellae* (fluoroquinolone-resistant), *Neisseria gonorrhoeae*, (cephalosporin-resistant, fluoroquinolone-resistant). Species under the medium list include: *Streptococcus pneumoniae* (penicillin non-susceptible), *Haemophilus influenzae* (ampicillin-resistant), *Shigella* spp., (fluoroquinolone-resistant).

Bacteriophage (phage) were first discovered in 1917 by d'Herelle, however their antibacterial activity was noted previously in 1896 by Hankin (Fenton et al., 2010). Microbiologists have been aware of the lytic activity of phage for over a century, and while the original concept was to use whole phage to control infection, and recently the lytic enzymes from phages are being explored for bacterial control of infections, with hopes of being a novel biologic therapy.

Lysin is a phage derived hydrolytic enzyme whose purpose is to lyse the bacterial cell wall via destruction of peptidoglycan as part of the lytic cycle for phage progeny release (Fischetti, 2009). Lysins have high specificity for their target bacterial cell as they bind to specific structures in the peptidoglycan for their cleavage action. Therefore, lysins cause minimal if any, destruction to the infected host's natural microbiome, due to their higher specificity for their target (Pottie et al., 2024), making them ideal candidates in theory for their application against antibiotic resistant bacteria.

Antibiotic resistance

As mentioned, antibiotic resistance is on the rise. A contributing factor with the current antibiotic regime in today's day and age is due to over-use in medicine (i.e., improper prescribing

of antibiotics, as well as patient compliance with completing the entire term of their prescribed time interval). Another factor is overuse in agriculture (i.e., antibiotic residues in animal-derived products for intensive farming in developing countries) (Loh et al., 2018). Over-prescribing can occur due to the inappropriate application of an antibiotic to an infection that doesn't require it based off the observed pathology in that the host has demonstrated ability to counter the infection. Also, over-prescribing can include the misapplication of the specific antibiotic to the infection at hand.

The antibiotic spectrum refers to the range of bacteria against which an antibiotic is effective. Narrow spectrum meaning that there is a narrow window of pathogenic bacteria to which it is effective against. Broad spectrum antibiotics have a much wider range and span of different types of bacterial infections to which they are effective against. If a prescriber possesses the inability to diagnose the bacterial pathogen for whatever reason, a broad-spectrum antibiotic is then selected and prescribed in anticipation of covering a larger spectrum of potential pathogens, rather than using a more narrow-spectrum antibiotic, which would increase the specificity for a specific species of bacteria. By doing this, opportunity of development of resistance for various bacteria increases due to improper exposure.

Historically, application of broad spectrum antibiotics mitigated the potential use of microbiological diagnosis, which could have helped prevent the production of antibiotic resistance (Melander et al., 2017). It's also worth noting that in the pharmaceutical industry, the focus on antibiotic discovery in its earlier stages revolved around searching for such broad spectrum antibiotics (Rex et al., 2014). Unfortunately using broad spectrum antibiotics can adversely affect the bacteria to which the host microbiota is comprised of, in that these bacteria can also act as hosts

for acquisition of resistance genes which remain for years within, and eventually can be transferred back to pathogenic bacteria, contributing to bacterial resistance (Melander et al., 2017). Also, another disadvantage to the application of broad spectrum antibiotics is its effects on the gut microbiota, in that antibiotic treatment can cause a compositional change within the microbiota affecting such processes as: nutrient supply, vitamin production and protecting from further pathogenic infections (Raymann et al., 2017). These effects can last up to two years post antibiotic administration, and can even result in permanent compositional changes of the microbiota.

Methods of acquisition of antibiotic resistance

i) Horizontal gene transfer

Resistance to antibiotics is typically due to the acquisition of novel bacterial resistance genes through various horizontal gene transfer mechanisms that include transformation, transduction and conjugation (Lerminiaux and Cameron, 2018). When bacteria acquire such resistance genes, it leads to novel resistant phenotypes, which ultimately results in antibiotic failure. Bacteria may acquire corresponding enzymes that inactivate antibiotics, and their corresponding genes may be present on plasmids and are highly transmissible between bacteria. Plasmids are a separate and extrachromosomal DNA element that replicate independently from the chromosomal DNA and can carry genes for antibiotic resistance. Bacteria can also acquire genes directly into their chromosomes, typically as mobile genetic elements such as transposons and integrons (Noel et al., 2022). Conjugation is a type of horizontal gene transfer between one host cell to another through the encoding of a sex pilus which is an extension that bridges from the donor host cell to the recipient cell by attaching to and drawing the recipient cell close for the transfer of the genetic material. During this process, a plasmid can then be transferred through this

pilus bridging system, potentially transferring genes for even multi-antibiotic resistance. Transduction refers to the introduction of bacterial genes during a virus infection cycle, where a bacteriophage virus had mistakenly packaged DNA from one species, and delivered these genes to another bacterial host species (Lerminiaux and Cameron, 2018).

Multiple plasmid-mediated mechanisms of resistance against the fluoroquinolones and aminoglycosides have been identified, and the combination of plasmid-mediated resistance with chromosomally encoded resistance mechanisms of multiple drug classes now presents the dilemma where strains that are resistant to all of the main classes of commonly used antimicrobial drugs (Schultz and Geerlings, 2012).

ii) Mutation

Alternatively, bacteria can develop resistance by acquiring specific mutations in their genomic DNA, which result in antibiotic targets that are no longer succumb to the antibiotic. Mutations can occur in topoisomerases leading to quinolone resistance, or in ribosomes leading to aminoglycoside resistance, or, mutations can be found in penicillin binding proteins that result in beta-lactam resistance (Egorov et al., 2018). Mutations are frequently acquired in outer membrane porins, which leads to altered porin structure that no longer permits the passive diffusion of antibiotics through the porin into the cell (Blair, 2015).

As already mentioned, an example of DNA mutational resistance results in the current emergence of specific Gram-negative bacteria that are resistant to expanded-spectrum cephalosporins (beta-lactam antibacterial) (Breijyeh et al., 2020). Such bacteria undergo a mutation which causes hyperproduction of their chromosomal class C β-lactamase (lactamases being enzymes that are produced by bacteria to evade antibiotic attack). This is considered the

main cause of resistance for *P. aeruginosa* clinical strains to not only cephalosporins but penicillins as well (Berrazeg, 2015).

Bacterial cell wall and differentiation

The bacterial cell wall provides essential structure for viability and protection against its surrounding environment. These structures are responsible for the shape of the bacteria as well as support and protection. It also provides receptor sites for viruses and target sites for antibiotics drugs (Rohde, 2019).

The Gram stain technique distinguishes bacteria based on the type of cell wall they possess. As Figure 1 below demonstrates: Gram-positive bacteria possess an outer surface-exposed, thick peptidoglycan layer in comparison to the Gram-negative bacteria, which possess a very thin layer of peptidoglycan, surrounded by an outer and inner membrane. Gram-negative bacteria have a glycolipid attached to their outer membrane called LPS, lipopolysaccharide, which is anionic. Gram positive have anionic polymers called teichoic acids within the peptidoglycan layers, and when anchored to the cytoplasmic membrane are referred to as lipoteichoic acids.

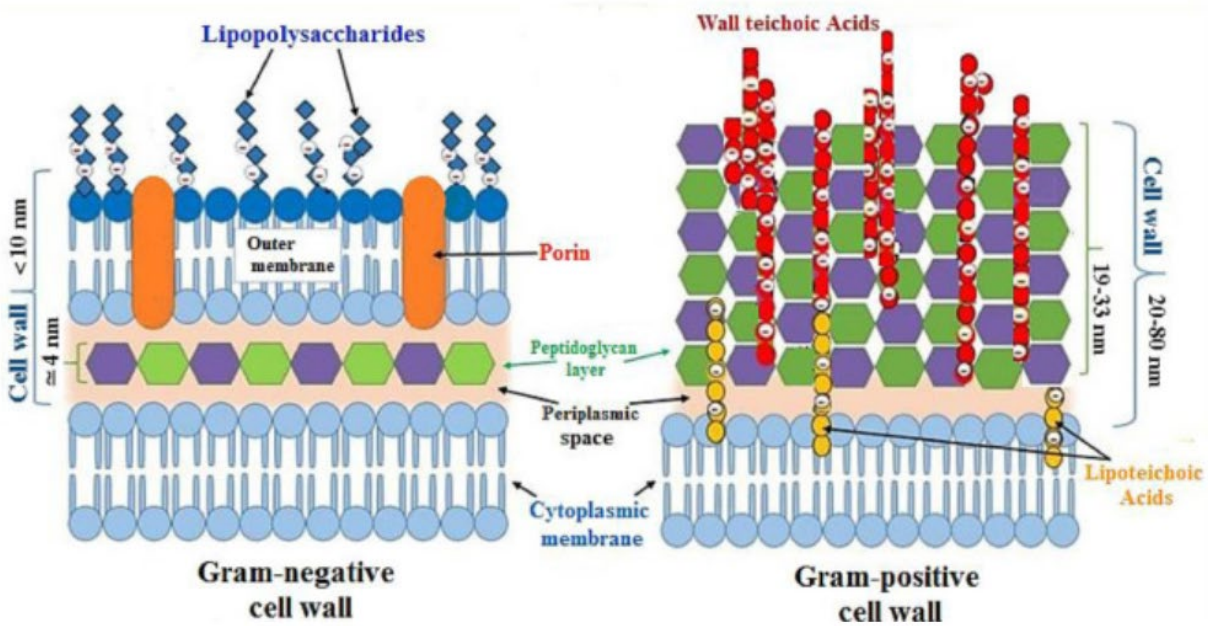


Figure 1. Structural differences of Gram-positive vs Gram-negative bacteria. Reprinted from Gram Negative Bacterium, an Overview from the Journal of Molecular Structure, K. S. Rizi, 2022, Elsevier. Volume 1262. [2022] by Elsevier. Gram Negative Bacterium - an overview | ScienceDirect Topics

Gram-positive bacterial membrane structure

Species of Gram-positive bacteria present in the "High" Category on the WHO's list of Priority Pathogens for Antibiotic Resistant Pathogens list include: vancomycin-resistant *Enterococcus faecium* and methicillin-resistant *Staphylococcus aureus* (as well as vancomycin-intermediate and resistant). Gram-positive bacteria have a distinct envelope and lack an outer membrane. However, like Gram-negative bacteria they also possess peptidoglycan and the peptidoglycan again is composed of repeating units of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc), also forming a netlike structure of crosslinks composed of about a dozen amino acids linked by peptide bonds. The combinations of amino acids present in the peptide stem of the peptidoglycan structure is dependent on the bacterial species (Vollmer et al., 2009). The main difference between Gram-negative bacteria peptidoglycan and Gram-positive peptidoglycan is that of the amount present and therefore its thickness. In Gram-positive bacteria

there are many more layers of peptidoglycan and its thickness is measured to be at 30-100 nm, whereas in Gram-negative bacteria it is quite minimal, and measured at only a few nanometers (Rohde, 2019).

Another difference is that Gram-positive bacteria have a corresponding structure to the LPS of Gram-negative bacteria; lipoteichoic acids (LTA). Lipoteichoic acids are polymers located within the cell wall and are considered a virulence factor with Gram-positive bacterial infections. These polymers are anchored to the cell membrane, and project through the peptidoglycan (Raymond, 2017). When lysis occurs with Gram-positive bacteria, the LTA is released and induces immune activation via TLR2-CD14.

i) Gram-positive antibiotic resistance mechanism examples

The mechanisms for antibiotic evasion for penicillin-resistant *Streptococcus pneumonia* and methicillin-resistant *Staphylococcus aureus* as well as vancomycin-intermediate and resistant includes: beta-lactamase production, altered penicillin-binding proteins, aminoglycoside-modifying enzymes, modification of the target site of the antibiotic and active efflux (Handzlik et al., 2013).

Regarding the mechanism of altered penicillin binding proteins, a serious bacterial resistant infection can occur with 'Methicillin resistant *S. aureus*' (MRSA). Methicillin is a narrow spectrum beta-lactam antibiotic. A narrow spectrum antibiotic is an antibiotic with activity against a limited range of bacteria, which differs from a broad spectrum antibiotic which has activity against a greater range of bacteria. Methicillin belongs to the penicillin drug family, whose mechanism of action is inhibition of the cross-linking action within the developing bacterial wall via penicillin binding proteins, ultimately preventing new bacterial cell wall formation. Therefore,

when the penicillin binding protein structure is altered in such a way where methicillin can no longer bind to these proteins, which renders methicillin useless and making such infections more difficult to treat bacterial infection (Siddiqui and Koirala, 2023).

***Staphylococcus aureus* is a model Gram-positive pathogen**

Staphylococcus aureus is a Gram-positive cocci bacteria, that can cause various types of infection, including skin lesions and localized skin abscesses, as well as furunculosis which can produce scarring (Ross and Shoff, 2023). Other types of infections include osteomyelitis, endocarditis, and food poisoning from release of enterotoxins into food. *S. aureus* is responsible for surgical wounds in the hospital setting and when combined with *Staphylococcus epidermidis,* can infect from indwelling medical devices (Foster, 1996). A toxigenic strain of *S. aureus* causes Toxic Shock Syndrome (TSS), which can be fatal. With TSS the superantigens that are produced from *S. aureus* bypass the normal immune pathway which activates T-cells, which causes an overwhelming cytokine response, ultimately over-amplifying the normal inflammatory response. With over stimulation of the inflammatory response, organ failure or even death can ultimately result, due to capillary leakage (Ross and Shoff, 2023).

Gram-negative bacterial membrane structure

Gram-negative bacteria possess an envelope around the cell which is made up of an inner membrane and an outer membrane. Between these two membranes lies the periplasm which is an area consisting of a thin layer of peptidoglycan. Peptidoglycan is the main component of bacterial cell walls and consist of repeating units of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc), as well as amino acids. These chains bind together to form a netlike structure of crosslinks composed of about a dozen amino acids linked by peptide bonds (Vollmer et al., 2008).

i) Lipopolysaccharide (LPS) and outer membrane permeability

Below in Figure 2 is a depiction of the LPS structure of Gram-negative bacteria. The overall composition and structure of the outer membrane of Gram-negative bacteria is widely conserved with little variation among different species (Vaara, 1992). The outer leaflet of the outer membrane of Gram-negative bacteria is comprised of lipopolysaccharide (LPS).

Lipopolysaccharide consists of O-antigen side chains which are attached to the core oligosaccharide segment which is composed of an outer-core component and an inner-core component and attached to the lipid A domain (hydrophobic region). Lipopolysaccharide structure varies between bacterial species. The immune response is dependent upon the structure of the Lipid A moiety. Therefore modifications that may occur to the Lipid A moiety would potentially assist the infecting bacteria with evasion of the immune system. O-antigens have demonstrated having the most variability of the LPS molecule, and are responsible for the virulence of the bacterial strain (Farhana and Khan, 2022).

Found within the outer membrane bilayer are β-barrel porin proteins, which are responsible for nutrient transport, modulation of antibiotic susceptibility and catalysis of specific enzymatic processes (Lee and Bayley, 2022). Underneath the outer membrane lies the periplasm to which the thin layer of peptidoglycan is located above the inner (or cytoplasmic) phospholipid bilayer membrane. While the outer membrane is an asymmetric lipid bilayer, with LPS on the outer leaflet, the inner membrane is a symmetric phospholipid bilayer (Gonzalez-Fernandez et al., 2022), containing anionic phospholipid head groups such as zwitterionic phosphatidylethanolamine (PE), and anionic phosphatidylglycerol (PG) and cardiolipin (Perczyk et al., 2020).

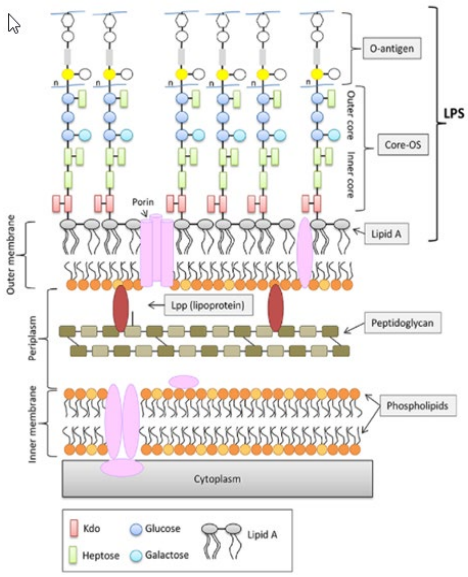


Figure 2. General Structure of the cell envelope of *Escherichia coli* K-12. Reprinted from Systematic view of the cell envelope of *E.coli* K-12, A., Ebbensgaard, et al., 2018, Volume 9. 108 by Frontiers. Frontiers | The Role of Outer Membrane Proteins and Lipopolysaccharides for the Sensitivity of Escherichia coli to Antimicrobial Peptides (frontiersin.org)

Lipopolysaccharide is a glycolipid on the surface of almost all Gram-negative bacterial cells that helps provides structure and acts as a permeability barrier to prevent many antibiotics from penetrating the bacterial cell wall and entering the cytoplasm to exert their antimicrobial activity. Because of this function, LPS and various LPS modifications that can occur, further reduce antibiotic entry, and are considered a main contributor for antibiotic resistance on behalf of many Gram-negative bacteria (Bertani, 2018). Such LPS modifications include fatty acid additions, phosphoethanolamine (PEtN) addition to the core and lipid A regions, 4-amino-4-deoxy-L-arabinose (Ara4N) addition to the core and lipid A regions, acetylation of the O-antigen and speculated possibility of hydroxylation of fatty acids (Gunn, 2001).

ii) Gram-negative antibiotic resistance mechanism examples

Gram-negative bacteria resistance includes different types of bacterial isolates and their unique resistance to varied antibiotic classes such as: quinolones, colistins (polymyxins), carbapenems, cephalosporins, and other β-lactam antibiotics (Breijyeh et al., 2020). To evade these specific antibiotic mechanisms of action, these bacteria acquire, evolve and share novel resistant phenotypes that ultimately lead to antibiotic failure. They may acquire corresponding enzymes that inactivate antibiotics, as well as mutations in native genes that permit antibiotic resistance. Obtaining the genetic blueprint to produce antibiotic resistance phenotypes is accomplished via acquisition of mobile genetic elements such as plasmids or gene islands, as well as via genomic mutation, both of which result in continual survival of the isolate (Munita and Arias, 2016).

One specific resistance mechanism is the current emergence of specific Gram-negative bacteria that are resistant to expanded-spectrum cephalosporins such as *E. cloacae, C. freundii, S. marcescens, P. aeruginosa.* These bacteria undergo a mutation which causes hyperproduction of their chromosomal class C β-lactamase (Tamma et al., 2019).

More specifically, AmpC β-lactamases are a specific type of chromosomal class C β-lactamases. These specific enzymes evade antibiotic attack towards not only 7-α-methoxy-cephalosporins such as cefoxitin or cefotetan, but also strains of carbapenems that are not in possession of membrane porins. This type of resistance has been demonstrated to cause nosocomial outbreaks, which are increasing in occurrence (Philippon et al., 2002).

Pseudomonas aeruginosa is a model Gram-negative pathogen

Pseudomonas aeruginosa is listed in the "critical" category of antibiotic-resistant bacteria of concern according to the World Health Organization. *P. aeruginosa* is a Gram-negative, rod-

shaped bacterium that infects humans, other animals and plants. It is generally considered an opportunistic pathogen that does not infect healthy individuals but is a major cause of infections in persons with a compromised immune system. For example, it is among the top three causes of all hospital acquired infections, such as patients on ventilators, or infections resulting from colonizing foreign implant devices, but also burn patients. It is the most common pathogen in severe burns and wounds, delaying the healing cycle due to infection (Raizman et al., 2021).

Cystic Fibrosis is an inherited disease caused by a mutation in the gene that encodes for cystic fibrosis transmembrane conductance regulator protein (CFTR), a chloride channel in lung epithelial cells. When mutation occurs in CFTR, it results in altered ion transport and leads to production of a hyper-viscous mucous on the airway epithelial surface. The mucous is colonized by microbes resulting in a chronic lung infection that causes lung deterioration and ultimately death. This disease also affects the liver, pancreas and kidneys. The thickening of the mucous in the respiratory pathway specifically causes the airways to narrow, making breathing quite difficult for affected patients. *P. aeruginosa* is also the 'major pathogen' in cystic fibrosis infections, which is complicated by the long-term survival strategies employed in the CF lung, and the stimulation of a chronic inflammatory response, both of which cause gradual lung deterioration (Reisin, 1994).

P. aeruginosa grow as a biofilm during infections in the respiratory tract. Biofilms can induce antibiotic resistance via the following methods: limited diffusion of antimicrobial agents through the biofilm matrix. Biofilm inducible resistance mechanisms, or increased tolerance through slow growth rates or persister cells (Hall and Mah, 2017).

Care management and treatment of cystic fibrosis requires that affected patients require lengthy and repeated hospitalization visits, often in response to an exacerbation. While traditional

therapy revolved around antibiotics, an attempt to control the bacterial infection, novel drugs that correct the misfolding of the CFTR protein are now frequently in use such as Trikafta which has been approved for and indicated for patients carrying deletion of phenylalanine at position 508(Veit, 2020). If Trikafta is applied to a patient who possesses the F508del mutation, it corrects the folding and presentation of the mature CFTR protein via folding correctors, and therefore CFTR function is improved. Currently the mechanism of Elexacaftor is not known (Veit, 2020).

The cost of "CFTR corrector" therapy with drugs such as Trikafta can cost up to $300 000 per year (Canadian Agency for Drugs and Technologies in Health, 2024), limiting its widespread usage.

Discovery of viruses that specifically kill bacteria

Bacteriophages, or commonly referred to as "phages", were first discovered in 1917 by d'Herelle, but their antibacterial activity was noted previously in 1896 by Hankin (Fenton et al., 2010). Due to the discovery of these viruses that specifically target and kill bacteria cells, a whole new field of antibiotic research has been developed, where the viruses are used as a direct substitute for an antibiotic. Historically, the whole phage itself had been used to control infection, an idea which is also gaining renewed interest.

Lysins are phage derived hydrolytic enzymes whose purpose is to lyse the bacterial cell wall via destruction of peptidoglycan in the cell wall as part of the lytic cycle for phage progeny release (Fischetti, 2009). Lysins have high specificity for their target bacterial cell, so in comparison to antibiotics which have a broader spectrum in terms of a target, lysins have higher specificity and cause minimal destruction if any, to the infected person's natural microbiome (Pottie et al., 2024).

When lysins are applied exogenously, they are effective against Gram-positive bacteria as their peptidoglycan cell wall is exposed. However, Gram-negative bacteria are covered by an additional outer membrane that protects the peptidoglycan from degradation by lysins, as the lysin mechanism of action is cleavage of the peptidoglycan wall, where location of the cleavage is dependent on the class of lysin. This cleavage action on the peptidoglycan structure causes lysis of the bacterial cells, bringing upon cell death. Here, I am interested in discovering lysins that may be useful as novel antibiotics against *P. aeruginosa*, a Gram-negative bacteria that has made the critical category of the WHO's Priority Pathogens list for antibiotic resistant pathogens.

Lysin Structure

Lysins are known to have a two domain structure which is made up of both a C-terminal domain and an N-terminal domain held together by a linker peptide, however the modular structure can vary between Gram-positive and Gram-negative bacteria (McGowan et al., 2012). The C-terminal domain is responsible for cell-wall binding action of the lysin, allowing it to bind to the peptidoglycan. The N-terminal domain is the catalytic domain to which the hydrolytic reaction of peptidoglycan cleavage occurs (McGowan et al., 2012).

The scenario for the catalytic structure for lysins is dependent upon their Gram-positive or Gram-negative designation. Gram-positive infecting lysins typically possess a modular structure consisting of either one or two catalytic domains, as well as a C-terminal cell wall binding domain. The cell wall binding domain is respective to its target bacteria. On the other hand, the majority of Gram-negative infecting lysins have a globular organization comprised of only one catalytic domain and usually don't possess a cell wall binding domain, but rather they possess terminally charged residues for binding (McGowan et al., 2012).

Recent studies have identified lysins that contain domains with features of antimicrobial peptides (AMPs), that allow lysins to target Gram-negative bacteria. In these cases, lysins can target their corresponding bacteria for destruction independently, without the necessity of being fused to another molecule, or requiring synthetic outer membrane permeabilizers to assist with their entry to disrupt the outer membrane (Heselpoth et al., 2022; Vazquez et al., 2022).

Antimicrobial peptides are short peptides that are amphipathic, meaning that they have hydrophobic and charged hydrophilic amino acids, which allow these peptides to bind and disrupt and destroy bacterial cells. For our research, one way for searching for such novel features within AMPs will be to assess the frequency of hydrophobic and charged amino acids, which includes a focus on tryptophan and arginine and their relative proportion to each other.

Categorization of lysin enzymatic activity

Lysins are classified into the following generalized categories based off of their cleavage site on the peptidoglycan structure to which they act upon, and these classes include: glycosidases, amidases, glucosaminidases and endopeptidases (Figure 3). Glycosidases are further divided into lysozymes/muramidases, lytic transglycosylases. Muramidases hydrolyze the alternating MurNAc and GlcNAc disaccharide backbone of peptidoglycan. Lytic transglycolases cleave the bond between alternating MurNAc and GlcNAc. Amidases hydrolyze the sugar and stem peptide bonds. Glucosaminidases work through the action of cleaving the glycosidic bonds of GlcNAc and their adjacent MurNAc in the sugar strand. Endopeptidases perform their cleavage action on the bonds between amino acids of stem peptides and the interpeptide bridges. (Ghose and Euler, 2020).

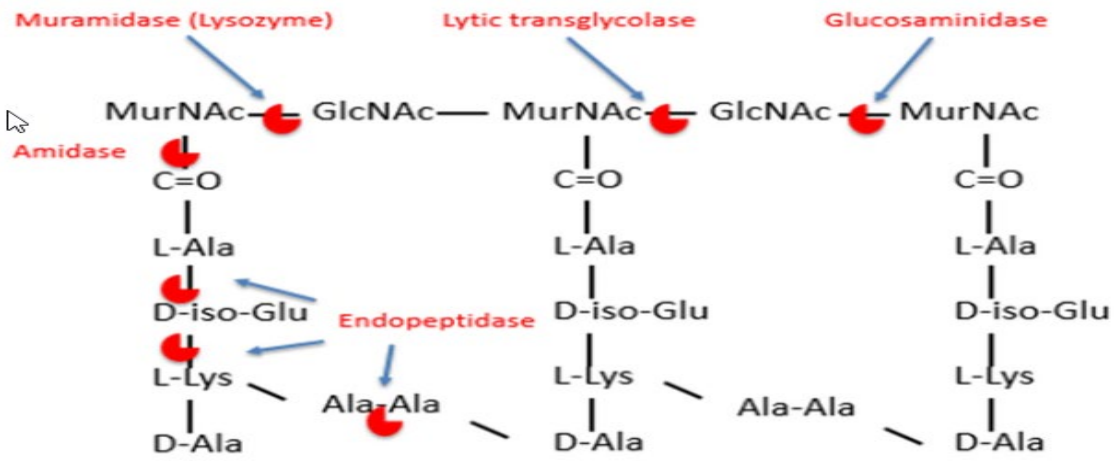


Figure 3. Lytic enzymes are categorized based on the type of the chemical bond that is cleaved within the peptidoglycan layer. Categories of lytic enzymes, Figure 1, reprinted from Gram-negative bacterial lysins, Ghose, C., Euler, C., 2020, 9(2)74 by MDPI: Antibiotics, Gram-Negative Bacterial Lysins (mdpi.com)

Table 1. Examples of lysins used as successful antimicrobials

Endolysin	Phage Origin	Enzyme type	Killing ability	Reference
CF-301 (also referred to as PlySs)	Prophage of *Streptococcus suis*	CHAP domain (large family of amidases) plus novel antimicrobial mechanism by activation of latent blood components (HSA and lysozyme) to facilitate more swift and effective antistaphylococcal activity in some species (i.e., humans, rabbits, and dogs) but not in others (i.e., mice and rats).	Complete removal of biofilms in catheters within 1 hour (demonstrated against S. aureus biofilms) ***is in clinical development for the treatment of *S. aureus* bacteremia and endocarditis	(Schuch, 2017; Bateman, 2003)

$CHAP_K$	*Staphylococcal* phage K	Endopeptidase	Complete elimination of *Staphylococcal* biofilms within 4 hours	(Keary, 2016)
PlyG	*B. anthracis γ* phage	Amidase	Kills *B. anthracis* isolates and other members of the *B. anthracis* 'cluster' of bacilli *in vitro* and *in vivo*	(Kikkawa 2008; Schuch, 2002)
CPl-1	*Streptococcal* phage CPl-1	Muramidase	Eliminated *pneumococci* (*Streptococcus pneumoniae* WB4) from blood (rats) within 30 min and decreased bacterial titers in vegetations (>4 log_{10} CFU/g) within 2 h.	(Entenza 2005)

The enzymatic diversity of lysins in terms of the various combinations of enzymatic domains they possess, creates the potential for engineering novel lysins with various combinations of binding and catalytic domains, and therefore further expands upon their potential utility on the therapeutic spectrum as a possible antimicrobial.

Holins disrupt the inner membrane and promote the activity of lysins

The lytic phage killing cycle is depicted in Figure 4 from Jose, 2018. Bacteriophages bind to the outer surface of Gram-positive bacteria by use of virion-associated lysins (VALs) which degrade the peptidoglycan, which is necessary for phage DNA to be then injected into the cell. Next, the bacteriophage genes are expressed, and then the bacteriophage proteins are produced within the host bacterium. During the late stage of the lytic cycle, holins are produced from the bacteriophage genome. Holins form pores within the inner membrane and this ultimately allows

lysins to reach the peptidoglycan layer, and the enzymatic action of lysins disrupts the peptidoglycan cell wall layer, to cause lysis of the cell (Saier and Reddy, 2015)

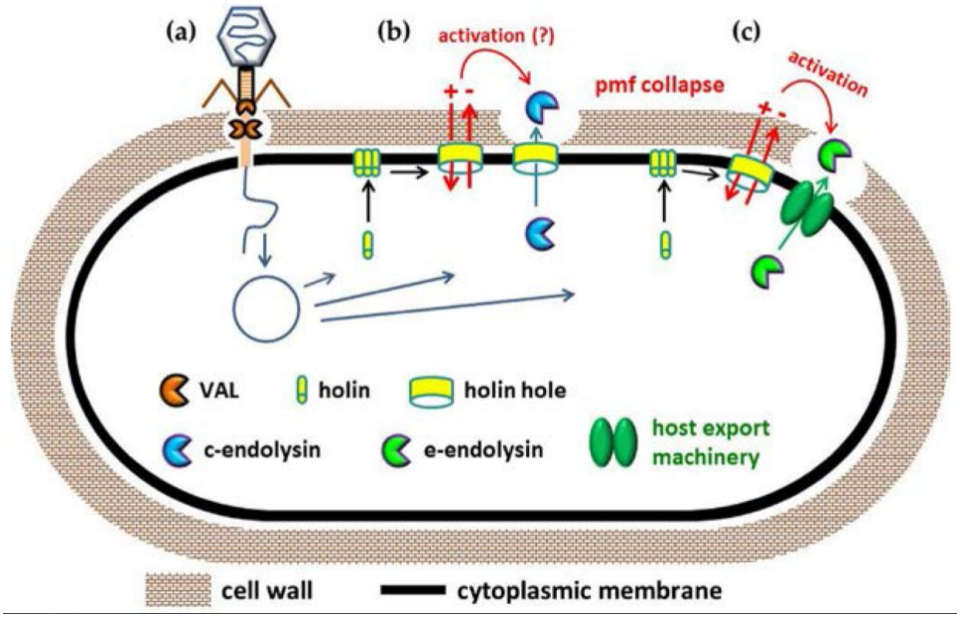


Figure 4. Lytic cycle of phage killing Gram-positive bacteria. Reprinted from Engineering of Phage-Derived Lytic Enzymes, Figure 1: natural context of action of phage lytic enzymes (PLEs), C. Jose, 2018, 7(2)29 by MDPI: Antimicrobials. Engineering of Phage-Derived Lytic Enzymes: Improving Their Potential as Antimicrobials.Virion-associated lysins degrade the cell wall after phage binding. After infection, different types of phage lysins such as canonical endolysins (c-endolysins) or exported endolysins (e-endolysins), depending on the pathway they follow to reach the CW, and are produced in addition to holin proteins. Some phages use host machinery to export lysins to access the cell wall. Most known endolysins are C-endolysins as their function has been defined by their ability to access the CW compartment through holin channels. Yet, there are some endolysins (e-endolysins) that are exported by way of host cell apparatuses, such as the bacterial Sec system, which is a crucial protein export pathway.

When lysin is biochemically engineered and introduced exogenously to Gram-positive bacteria, the speed of lysis is increased compared to what it is when it is performing its function from within the cytoplasm, as there is no interference present from the plasma membrane of the bacterial cell, and therefore holin is not required (Fenton et al., 2010). Holin itself isn't activated through a signal transduction cascade, but through genetically determined expression time in the lytic cycle (Fenton et al., 2010). From the findings in a study by Gründling et al. (2001), it was suggested that an infected bacterial cell membrane's disruption was due to the formation of an

oligomeric complex that was the product of accumulated critical levels of holin, produced by the infected bacteria of which the bacteriophage expressed its DNA (Gründling et al., 2001).

In a 2018 study conducted by Guo et al., a higher level of the factors surrounding holin and its' functionality were observed through the application of the *Lactobacillus fermentum* phage ϕ PYB5 with its conjugate bacteria, *Lactobacillus*. The exact timing of lysis initiation is regulated by such proteins as holin inhibitors or antiholins. The Hyb5 gene within this phage encodes the holin protein which is a transmembrane protein with an N-terminal transmembrane domain and a C-terminal cytoplasmic domain. When 45 amino acid residues sequences were deleted of hyb5 of the C terminus region (which normally consists of 154 amino acid residues) early lysis of the cell occurred indicating that this specific region of the C terminus region is required for binding of the antiholin to keep the system intact and to place a "hold" order on the holin protein itself to prevent early lysis in order to maximize viral progeny production within the host cell (Guo et al., 2018).

Considerations for an ideal antibiotic

Under ideal conditions, an antibiotic would need to be successful at efficiently killing the pathogenic bacteria, without significantly disrupting the host's natural microbiome. This pathogen selectivity would minimize the potential for secondary infection based upon opportunistic growth of specific microorganisms that constitute the natural microbiome. Ideal antibiotics therefore require the need to remain stable and active upon delivery to the target site or tissue with the mode of administration selected (Leekha et al., 2011). Lastly, the ideal antibiotic would need to demonstrate that it does not select for antimicrobial resistance during the course of treatment (Leekha et al., 2011).

Lysin as a potential antimicrobial

When investigating a potential biologic for therapeutic intervention, specific parameters must be considered beforehand to assist with establishment of benefit versus risk to the potential subject species of which the therapeutic would be applied to. Below, a few significant parameters are discussed based on previous studies conducted around this subject matter: toxicity, immunogenicity, resistance and synergy.

i) Toxicity: studies in animal models

SAL200 (more recently known as LSVT-1701) is a candidate anti-staphylococcal phage lysin antimicrobial therapeutic. It was assessed by Jun et al. (2014) for potential toxicity effects in both rodents and dogs. SAL200 is comprised of recombinant phage endolysin SAL-1, which has previously demonstrated a lack of toxicity when administered intravenously to both rats and dogs. Other than a minor transient change in cardiovascular function which resolved in six hours post administration, and some vomiting and subdued behavior that resolved in 30-60 minutes post administration, no significant changes to body weight, food consumption, electrocardiography, urinalysis, hematology, serum biochemistry occurred; indicating a lack of toxicity following administration (Jun et al., 2014). When administered to Sprague-Dawley rats both single and repeated-dose toxicity studies indicated no sign of toxicity. No rats died during this test. As well, none of the rats developed abnormal clinical signs including abnormal necropsy findings, or abnormal weight changes. Doses were documented as well tolerated, contributing to the safety profile of this potential antibiotic (Jun et al., 2014).

Application of SAL200 (LSVT-1701) was used in clinical trials with humans and it was considered safe and well tolerated and no serious adverse effects or deaths occurred. Treatment-

emergent adverse events (TEAEs) were stated to be of mild intensity (Wire et al., 2022). The most common of the TEAEs were chills, pyrexia, headache, infusion site events, cough, rhinorrhea, and increases in C-reactive protein. Since lysins are proteins not produced by the animal host, they could induce an immune response. C-reactive protein (CRP), is a non-specific inflammatory marker produced by the liver and found in the bloodstream and was used for identification for any increase in inflammation, which can be a direct response to infection or immune response (Wire et al., 2022).

ii) Immunogenicity

Lysins are not native proteins of the animal and if applied as an enzybiotic could therefore elicit an immune reaction, such as an allergic response.

In the rodent study, anti-SAL-1 antibodies were not detected in the blood samples collected on day 14 but were detected on day 28 samples during the related repeated-dose study in the rats (Jun, 2014). On day 42 during the recovery period, the antibodies were only slightly higher as compared to day 28. Whereas in the repeated-study for the dogs, anti-SAL-1 antibodies were detected on day 14 in all groups and like the rats, the amount of antibodies only slightly increased during the recovery period. This then could be concluded that the SAL-antibody production was induced from repeated administration of SAL200 for longer than one week. This could potentiate an immune response should re-administration of SAL200 happen again.

A study conducted by (Harhala et al., 2022) observed a similar trend with mice that were administered the lysin PlyC. Immunoglobulin G (IgG) measurements were performed over a seventy day period. PlyC did induce an IgG response and its' increase commenced on day 15, with the highest amounts observed approximately 30 days after administration. However, these authors

performed further analysis and noted that the immunogenic regions of the PlyC lysin had two subunits including: PlyCA and PlyCB, and the immunogenic regions that IgG interacted with was all within the PlyCA unit (Harhala et al., 2022).

Due to the occurrence of immunogenicity, this is an area that would require further study for lysins, because they may be modified for improvement to avoid immunogenicity, so that the likelihood of the host to experience an allergic reaction is little to none. Also, there is the potential that a person may become immune to the lysin therapy if they were issued the same lysin therapy repeatedly.

It has been demonstrated that in systemic infection application, the host immune response can be lessened by conjugation of the lysin protein to polyethylene glycol (PEG) which reduces the ability of the host antibody to bind to the lysin protein, even though a slight decrease in lysin activity was noted in this circumstance (Fenton et al., 2010). However, in the previously mentioned studies, lysin was not significantly eradicated from the host system indicating that its' activity was not significantly hindered or halted; indicating once again their potential application for antibiotic resistant infections (Fenton et al., 2010).

iii) Resistance

Resistance is an important consideration when developing an antibiotic because it must avoid developing an acquired resistance after repeated administration to maintain their efficacy over time.

Previous studies have indicated that when *Streptococcus pneumoniae* was repeatedly exposed to low concentrations of lysin Pal on agar plates, resistant strains of the bacterium did not develop (Fenton et al., 2010). *Bacillus cereus* was also repeatedly exposed to its respective lysin

PlyG at low concentrations in liquid culture, and this bacterium also did not develop any resistant strains (Fenton et al., 2010). *B. cereus* also demonstrated that even with an antibiotic resistant isolate of the bacterium (that possessed a 1000 to 10,000-fold increase in antibiotic resistance through metagenesis with methanesulfonic acid ethyl ester) it remained sensitive to the lysin PlyG without the presence of any mutant strains developing towards the lysin (Fenton et al., 2010). Due to the fact that the bacterial cell wall is highly conserved amongst bacterial species, resistance formation towards lysins in particular would prove to be difficult.

Since Gram-positive lysins have two catalytic domains where each domain has a different specificity for peptidoglycan, this creates the opportunity for the enzyme to target multiple modes of peptidoglycan breakdown, which could help prevent resistance from forming (Fischetti, 2009).

iv) Synergy

Different lysins have demonstrated the capability to have a synergistic effect when applied together. An example of this is demonstrated in a study performed by Loeffler and Fischetti (2003) using *Streptococcus pneumonia* as the pathogenic bacteria. The Cpl-1 lysin which is a muramidase derived from pneumococcal phage, and the Pal lysin which is a lysozyme derived from pneumococcal phage, were the lysins of interest utilized in this study. Pal and Cpl-1 were tested separately for their killing activity and then combined to determine the lytic activity. When combined, the lysins demonstrated an enhanced killing activity against *S. pneumonia* then each on their own separately. These two types of lysins cleave the peptidoglycan wall at different sites, as they have different N-terminal catalytic sites but the same C-terminal cell wall attachment site which binds to choline. In 30 s, 1 U of Pal per ml reduced the bacterial titer of the *S. pneumonia* by a median of 1.34 $\log_{10}$ CFU/ml while Cpl-1 at 1 U per ml reduced the titers by 0.83 $\log_{10}$

CFU/ml. The combination of both lysins reduced the bacterial titre by 2.4 $\log_{10}$ CFU/ml. After 10 minutes the combination of both enzymes was 3.15 $\log_{10}$ CFU/ml compared to Pal alone which was 1.99 $\log_{10}$ CFU/ml and Cpl-1 alone which was 1.44 $\log_{10}$ CFU/ml (Loeffler, 2003). Lysins destroy the peptidoglycan directly, killing both growing and non-growing cells (Matsukzaki et al., 2005) whereas antibiotics such as penicillin and cephalosporin's mechanism of action includes inhibiting peptidoglycan synthesis, thereby destroying only dividing cells.

v) Route

The administration route of a protein antimicrobial is important. Since lysins are proteins, and are therefore susceptible to denaturation, oral administration would not be ideal due to the low pH, digestive enzymes (i.e., trypsin, chymotrypsin, pepsin and peptidase) and mechanical digestion that exists in the stomach, which would denature lysin proteins (Patricia and Dhamoon, 2022). This denaturation would reduce the efficacity of lysin due to irreversible damage of the protein, also decreasing its bioavailability. In studies thus far, administration has most commonly been through intravenous (IV) injection which aids with the maintenance of protein structure upon entry and increases the protein's bioavailability (Murray, 2021) as it can reach the bloodstream without being denatured by the GI system, if administered orally.

Lysins have shown promise with skin infection studies involving the mouse model and skin that had been infected with *P. aeruginosa*. In a study conducted by Raz et al. (2019), mice were shaved and then inoculated with 10 µl of log-phase *P. aeruginosa* PAO1 at a concentration of 5 x 10^6/CFU/ml. After 20 hours of allowing the infection to initiate, mice were topically treated with 25 µl doses of lysin PlyPa03 in CAPS-buffered saline. PlyPa03 demonstrated noteworthy killing of *P. aeruginosa* with a >2 $\log_{10}$ kill (Raz et al., 2019).

Another potential route for administration could be nasal, although bioavailability may be more limited due to the decreased surface area present for drug absorption. The lysin protein molecule's size would then need to be considered as well because any protein over 1000Da in molecular weight is proposed to encounter difficulty when crossing nasal epithelia (Ozsoy et al., 2009). Since lysins on average range from 15-40kDa, this molecular weight may be problematic when considering this as a consistent route as a potential for drug construction (Murray et al., 2021).

To avoid potentials denaturation and irreversible protein damage through oral route administration, and to provide alternatives modes of delivery other than to the IV administration method, research is branching into other potential modes of drug delivery.

Nanoparticles which are small, micro-scale particles within the range of 1-100 nanometers (nm), have been considered for delivery as they are able to encapsulate protein-based drugs, protecting them from the changing surrounding environment within the host's system until they reach their target destination. Also because of this mode of preservation, a more specific and dependable drug dose amount can be delivered as well (Murray et al., 2021).

Unique modes of lysin application to treat Gram-negative bacterial infection

Gram-negative bacteria presents a challenge when being treated with lysins due to the outer membrane protecting the peptidoglycan. Various studies have engineered lysins to encode additional antimicrobial protein-like domains to assist with permeating the outer membrane (Carratala et al., 2023; Heselpoth et al., 2019; Zampara et al., 2020). These engineered modifications include extra polycationic domains or proteins that carry a positive charge which disrupts the Mg^{2+} and Ca^{2+} divalent cations that cross-link the LPS molecules together, as well as

possessing hydrophobic activity which assists with entry across the outer membrane asymmetric lipid bilayer of the Gram-negative bacterial cell wall (Carratala et al., 2023). These methods will be briefly discussed in the subsections below.

i) Artilysins

Artilysins are a type of artificially modified lysin where an endolysin is fused to a peptide that consists of positively charged amino acids at one of the terminal ends (Carratala et al., 2023). The positively charged amino acid sequence on the terminal ends is added to destabilize LPS (which is normally stabilized through ionic interactions between divalent cations and phosphate groups) on Gram-negative bacteria. Briers et al., demonstrated successful development of artificially modified lysins that could permeate the barrier of Gram-negative bacteria, such as *Pseudomonas aeruginosa* strain PAO1, using endolysin OBPgp279 (originating from *Pseudomonas fluorescens* phage OBP) fused to a polycationic nonapeptide (PCNP). This engineered artilysin denoted as LoGT-001, exhibited a 2.61+/- 0.09 $\log_{10}$ reduction of antibacterial growth against *P. aeruginosa* PAO1 in just 30 minutes (Briers et al., 2014).

ii) Lysocins

Lysocins are a class of bioengineered proteins whose structures are composed of a bacteriocin fused to a phage-derived lysin with the aim of permeating the Gram-negative cell wall outer membrane. Bacteriocins are robust antimicrobial peptides which are produced by bacteria to kill or inhibit the growth of closely related bacterial strains, and these such peptides do not harm the bacteria that produces them due to the production of immunity proteins (Yang et al., 2014). This type of potential protein is unique due to the synergistic combined mechanisms of the bacteriocin and the lysin.

In a 2019 investigational study conducted by Heselpoth et al., they combined the PyS2 lysin with a colicin-like bacteriocin (S-type pyocin) of *P. aeruginosa*. This lysocin PyS2-GN4 allowed the translocation of the lysin through the outer membrane of *P. aeruginosa* with minimal LPS endotoxin release. It also displayed a narrow-spectrum of antipseudomonal activity in human serum, disrupted biofilms, and demonstrated no toxicity to eukaryotic cells (Heselpoth et al., 2019).

iii) Innolysins

Innolysins are another engineered lysin created for application against Gram-negative bacteria. Lysins with their enzymatic affinity for the peptidoglycan layer within the bacteria were combined with phage receptor binding proteins to enhance binding to Gram-negative bacteria. In a study conducted by Zampara et al., different phage endolysins were used in combination with receptor binding protein (RBP) Pb 5 in different configurations. The combination approach with different phage lysins allowed for customization of the endolysins for targeting different Gram-negative bacteria types such as *Pseudomonas aeruginosa*, *Shigella sonnei*, and *Escherichia coli* (Zampara et al., 2020).

The role of cationic peptide domain of successful Gram-negative lysins

Different molecules have been fused to lysins to assist with delivery across the Gram-negative outer membrane permeability barrier, in order to reach the peptidoglycan. In non-capsulated bacterial species such as *Pseudomonas*, the glycolipid LPS is exposed at the cell surface which is different than capsulated strains, where LPS is present below the capsule layer. Capsulated bacteria are bacteria species that have an outer layer compromised of polysaccharides that borders the cell wall, and is involved with survival as well as pathogenicity (Gao et al., 2024).

The inner leaflet of the outer membrane is composed of phospholipid (Clifton et al., 2015). The O-antigen component of LPS is the repeating hydrophilic distal oligosaccharide, which is the component of the LPS molecule that is directly exposed to the surrounding outer environment. Stabilization of LPS is achieved by divalent cations that decrease the overall negative charge to the membrane, such as Mg^{2+} and Ca^{2+} (Farhana and Khan, 2022).

To permeabilize the outer membrane, these cations can be destabilized by physical cation chelation with compounds such as citric acid, and ethylenediaminetetraacetic acid (EDTA), or with the solvent trichloroethane ($CHCl_3$), or the detergent Triton X-100 (Khan et al., 2021). The use of cation chelators has been documented in experimental studies with permeability assistance of lysins to help them cross the outer membrane of Gram-negative bacteria (Khan et al., 2021). However, due to their cellular cytotoxicity, chelators are not viable options for fusion-lysin molecules (Khan et al., 2021). However this information has assisted with the overall understanding of what is likely to be required in addition to the lysin when considering novel antimicrobials to treat a Gram-negative bacterial infection.

Antimicrobial Peptides

Antimicrobial peptides (AMPs) are small peptides that have direct killing activity against bacteria. They target microorganisms for destruction and exist in a variety of different settings. In humans, AMPs function within the immune system to assist with immune-mediated responses to control infection by destroying respective microbes. Previous research states that these AMPs on average have a net charge between +2 and +9, and are amphipathic (Haney et al., 2019).

Importantly for this work, there have been a few lysins that have demonstrated the ability to work independently on permeabilizing the outer membrane of Gram-negative bacteria without

any assisting membrane disruptor solvent or fused AMP suggesting that the lysin had an embedded AMP-like domain within its sequence (Vazquez et al., 2022; Heselpoth et al., 2022). These studies have characterized lysins that were demonstrated to have built-in antimicrobial peptide regions within their catalytic domain.

According to a study performed by Vazquez at al., (2022) Pae87 which is a lysin derived from *Pseudomonas aeruginosa* phage JG004, demonstrated 2 $\log_{10}$ killing for PaO1 as well as 1-3 $\log_{10}$ killing of a range of six other *Pseudomonas aeruginosa* strains tested (Vazquez, 2021). Pae 87 had mild activity with ≤1 $\log_{10}$ killing against *M. catarrhalis*, and good activity with 1–2 $\log_{10}$ killing against *Acinetobacter baumannii* (Vazquez, 2021).

Vazquez et al., later demonstrated (2022) that lysin Pae87 had a separate substrate-binding region within the catalytic domain 18Å from the catalytic site and located on the opposite side of the lysin molecule. The authors stated that the enzymatic destruction of the peptidoglycan was unrelated to the antimicrobial activity of Pae87 against the outer membrane of Gram-negative bacteria, in that a separate antimicrobial peptide-like region within the C-terminus of the Pae87 then classified as P87, was responsible for this independent action (Vazquez et al., 2022).

Heselpoth et al., extracted and modified protein PaP1 from the C-terminal end of the PlyPa01 lysin, which had also previously been studied for killing ability with PaO1 (Raz et al., 2019). This was done on the basis that PlyPa01 possessed a coterminal end that was compromised of AMP-like characteristics (Heselpoth et al., 2022). PaP1 demonstrated a killing ability of 4.3 to >5 $\log_{10}$-fold for the stationary growth phase and log phase respectively, for against PaO1. Results also demonstrated that this protein killed bacteria upon contact and it held stability over a wide temperature and pH range, as well as autoclaving. However, it was sensitive to high salt

concentrations because this inhibited its function. Importantly, this protein lacked cytotoxicity toward human cells, which is essential when considering it for use as a potential antibiotic for humans (Heselpoth et al., 2022).

Characteristics of Antimicrobial Peptides

Most AMPs are short peptides that range from 10 – 60 amino acids in length (Yuan et al., 2020; Huan et al., 2020). Two features commonly associated with having AMP activity are the presence of both cationic and hydrophobic amino acid residues in high proportions (Huan et al., 2020). Some research has specified that the AMP's average net charge is 3.32. However, several anionic AMPs exist which have an overall net negative charge such as the human anionic AMP dermcidin (Lai et al., 2006).

Classification of Antimicrobial Peptides

Antimicrobial peptides are classified based on their source, activity, structural characteristics and amino acid quantity. Possible sources of AMPs include peptides derived from mammals, plants, amphibians, microorganisms and insects (Huan et al., 2020). The activity of AMPs is classified by function of the antibacterial peptide which includes: antibacterial peptides, antifungal peptides, anti-inflammatory peptides, antiviral peptides, antiparasitic peptides and anticancer peptides (Huan et al., 2020). The structural characteristics of AMPs refers to their protein structure (α-helix, β-pleated sheets, linear extension structure and proteins with a combination of α-helix and β-pleated sheets).

Proposed mechanisms of outer membrane disruption for AMPs

Differing modes of membrane disruption by AMPs depend on their physical properties and the host cell membrane configuration. Research has proposed certain models of outer-membrane disruption such as the barrel and stave model, the carpet model, and toroidal pore model as shown in Figure 5. Membrane disruption through permeabilization is vital for allowing the AMPs to translocate to the bacterial cytoplasm (Huang and Li, 2023). Although the following Figure 5 represents AMP permeabilization on a single bilayer to eventuate to the bacterial cell's cytoplasm, this is only a representation for Gram-positive bacteria, because the AMP must permeate both bilayers of the Gram-negative bacterial membrane to reach the cytoplasm for Gram-negative bacteria should the cytoplasm be its final destination (Scocchi et al., 2011). Various theories exist around possible different mechanisms of translocation to the cytoplasm after permeabilization, such as AMPs that are rich in proline that translocate into the cell via formation of transient pores which allow them to get to their intracellular targets (Scocchi et al., 2011). In addition, other research has speculated that some AMPs that have the ability to cross bacterial membranes through receptor-mediated transport and may possess the ability to target intracellular process such DNA replication, transcription and translation (Otvos, 2002; Ulmschneider, 2017; Friedrich, 2001). In addition, Nisin, is an AMP that has demonstrated the ability to bind to Lipid II, which is a precursor for peptidoglycan synthesis through what is considered as a "receptor-mediated" mechanism, as the Lipid II molecule possesses a negative charge which attracts the cationic AMP (Breukink et al., 2023).

In terms of Gram-negative bacteria, disruption of the outer lipid-bilayer would be sufficient enough for cell death purposes as it would decrease its overall stability causing the bacterial cell to lyse.

As shown in Figure 5, the carpet model reflects an accumulation of AMPs on the surface of the membrane and attacking in a "detergent" like activity with electrostatic attractions between the acidic, lipid-rich areas of the Gram-negative bacterial outer-membrane (Huan et al., 2020). The AMPs are said to 'carpet' the bacterial surface. The barrel stave model, perhaps a less common mechanism, depicts AMPs aggregating together, then inserting into the membrane bilayer in a parallel fashion to the existing phospholipids to form a channel. This model is often affiliated with hydrophobic peptides bearing 20 amino acids or greater. In the toroidal model, the AMPs accumulate vertically and embed themselves in the membrane and then conform to create a ring hole; also known as the worm hole model (Huan, et al., 2020). Here the hydrophilic section of the AMP interacts with the phospholipid head groups of the outer and inner leaflets of the lipid bilayer membrane continuously (Huang and Li, 2023; Huan, 2020).

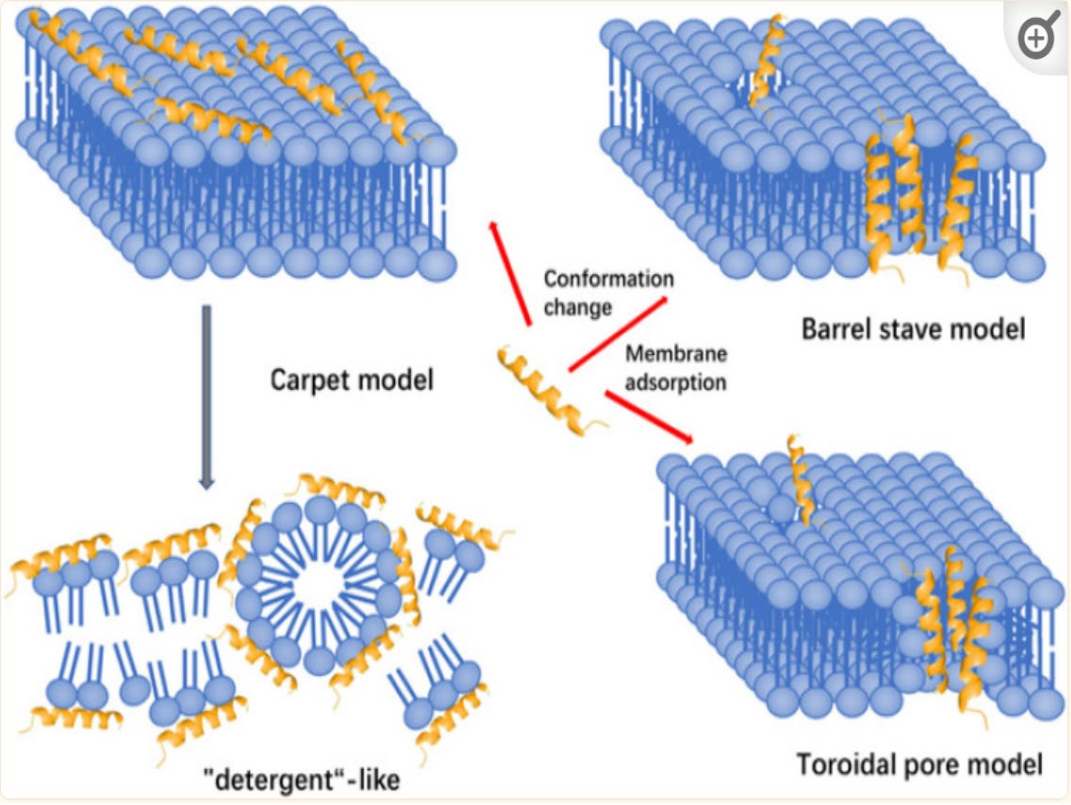


Figure 5. Models of action for extracellular AMP activity. Reprinted from Antimicrobial Peptides: Classification, Design, Application and Research Progress in Multiple Fields, Y., Huan, et al., 2020, Volume 11 by Frontiers. Frontiers | Antimicrobial Peptides: Classification, Design, Application and Research Progress in Multiple Fields (frontiersin.org)

Some research has pointed out that some AMPs are able to translocate into cells to facilitate their activities without destroying the cells themselves, such as with immunomodulatory host defense peptides, (HDPs) (Haney et al., 2019), and as such, fall into the class of cellular penetrating peptides (CPPs). Cellular penetrating peptides are described as short amino acids that often have a positive net charge, and are able to penetrate cell membranes. Because of this, there are suggestions that some AMPs can do the same by accumulation in the cytoplasm of bacteria (Haney et al., 2019).

Proposed mechanism of the outer-membrane disruption for *Pseudomonas aeruginosa*

Antimicrobial peptides can displace the divalent cations such as Mg^{2+} and the Ca^{2+} bound to the LPS molecule, which destabilizes LPS and ultimately causes a pore on the outer membranes of bacteria. This allows the AMPs to enter through the outer membrane and to eventually bind to the charged phospholipids on the inner cytoplasmic membrane (Heselpoth et al., 2022).

Arginine and Tryptophan

Assumptions have been put forward in regards to the necessity of the presence of specific amino acids such as arginine and tryptophan, within AMPs. In some ways AMPs are speculated to behave similar to CPPs. In the past CPPs and AMPs have been reviewed together as membrane-active peptides (Henriques et al., 2006). Both are short peptides in length ranging on average between five and fifty amino acids. They are generally cationic in nature and are amphipathic with hydrophobic residues, such as tryptophan, within their amino acid sequence. Both CPPs and AMPs

can interact with cell membranes, however CPPs are able to interact with constituents of eukaryotic membranes, whereas AMPs generally interact with bacterial cell membranes. These groups of membrane-active peptides are further differentiated by their means of application as CPPs are mostly involved with drug delivery such as gene therapeutics and cancer therapeutics, whereas the function of AMPs is to extinguish microbial activity (Macyszyn, 2023).

According to research, AMPs and CPPs rich in both arginine and tryptophan were found to have the highest antimicrobial activities (Chan et al., 2006; Walrant et al., 2020). Arginine allows for displacement of the divalent cations of Mg^{2+} and Ca^{2+} of the LPS molecules, disrupting their crosslinking, allowing further access to the outer-membrane asymmetric lipid bilayer. (Chan et al., 2006). Also, is a wide acceptance of a theory that arginine's guanidinium-bearing side chains have an important role in regards to recognition of negatively charged ions of lipids through electrostatic interactions and formation of bidentate hydrogen bonds with the gram-negative phospholipid asymmetrical outer membrane (Chan et al., 2006; Walrant et al., 2020) as shown in Figure 6 below. Arginine is a basic amino acid that issues positive peptide charge and hydrogen bond interactions, which are essential properties to combine with the bacterial membrane's abundant anionic component of phosphatidylglycerol (PG) and cardiolipin (CL).

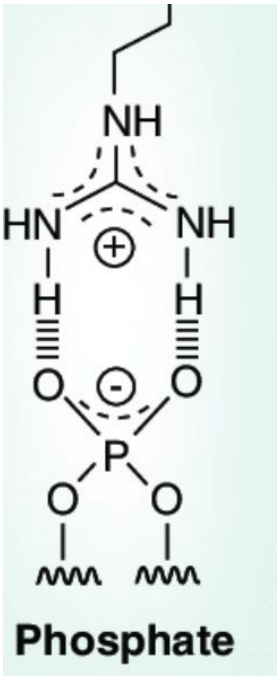


Figure 6. Hydrogen bidentate bond. Reprinted from Molecular Partners for Interaction and Cell Internalization of Cell-Penetrating Peptides: How Identical Are They? A., Walrant et al., 2011, by Nanomedicine. Molecular Partners for Interaction and Cell Internalization of Cell-Penetrating Peptides: How Identical Are They?: Nanomedicine: Vol 7 , No 1 - Get Access

The relevance of the component of tryptophan in this study is its speculated role in concert with arginine-rich AMPs as a natural aromatic activator through cation-π interactions, in turn enhancing the peptide to outer membrane interactions (Walrant et al., 2020; Chan et al., 2006). Also, tryptophan side chains have been associated with peptide folding in aqueous solution through maintenance of native and nonnative hydrophobic contacts, helping to achieve a conformation that best allows for alignment with the bacterial membrane for eventual pore creation for AMP entry (Chan et al., 2006). Previous research has suggested smaller peptides ranging from 5 – 11 amino acids with a minimum of three tryptophan and three arginine residues that were important towards *P. aeruginosa* antimicrobial activity (Strom, 2002).

For this research we propose that lysin regions rich in arginine will allow the arginine to participate in displacing the divalent cations of Mg^{2+} and Ca^{2+}, as well as allowing arginine to bind with the charged phospholipid region of the outer membrane through hydrogen-binding of its guanidinium side chain. We also believe that tryptophan may assist with the lysin's entry across the outer membrane due to its hydrophobic nature which is said to assist with orientation of the protein to align with outer membrane for eventual pore formation. If true, it would ultimately give the lysins access to the periplasm where the peptidoglycan layer could then be degraded.

Research Hypothesis

Due to the availability of advanced DNA sequence technologies, the field of microbiology has been radically transformed, and has led to the generation of many complete viral and bacterial genome sequences. Sequencing DNA used to take years, but now it simply takes a matter of minutes (Hall, 2007). Over 1 million high quality bacterial genomes have now been sequenced, including 4 billion genes from 40 000 bacterial species (Fullam, 2022). For this project, we will exploit the availability of numerous phage genomes that are contained within bacterial genomes. We will take advantage of The Phage Lytic Proteins database (PhaLP) database, which catalogs all predicted lysin sequences within all bacterial genomes. Our studies will be limited to *P. aeruginosa*, and *S. aureus*, to examine one Gram-negative and one Gram-positive organism respectively.

The research hypothesis is that lysins from bacteriophages that target the Gram-negative pathogen *P. aeruginosa* will possess greater features of antimicrobial peptides within their coterminal sequences, such as cationic charge, hydrophobicity, and amphipathic domains with specific amino acid quantities, that would promote disruption of the outer membrane and enhance

lysin antimicrobial activity when compared to lysins that target the Gram-positive pathogen *S. aureus*.

Materials and Methods

Introduction

A bioinformatic approach was used for the application of this study. Bioinformatics is a scientific discipline of analysis that uses a combination of biology or biomedical science with statistics and computer science for its analysis and delivery (Moore, 2007). Through its methodology it provides management, analysis and interpretation of data from previously conducted biological experiments as well as observational studies (Moore, 2007). It therefore facilitates implementation, data analysis as well as data mining from the exhaustive number of bacterial genome sequences available (Moore, 2007).

Research purpose

The purpose was to investigate whether or not a strong relationship exists between amino acid charge, hydrophobicity and the amounts of arginine and tryptophan present in the 50 amino acid C-terminal end of all non-redundant *P. aeruginosa* lysins extracted from the PhaLP database, and to predict their protein structure, and to ultimately propose a refined group of candidates based on specific criteria for future testing of this hypothesis. A direct comparison was made between *S. aureus* and *P. aeruginosa* to test our hypothesis.

Specific criteria of antimicrobial peptide-like sequences

The characteristic criteria that needs to be considered when designing AMPs are size, residue, composition, overall charge, secondary structure, hydrophobicity and amphiphilic

character (Fjell, 2012). For this study, we identified lysin sequences with antimicrobial peptide-like domains by considering their peptide length, location within the protein, presence of charged amino acid content, the specific quantities of arginine and tryptophan in relation to each other, hydrophobicity, secondary structure (helical), amphiphilic character, and Uniprot enzyme classification type.

The length of 50 amino acids was selected based upon previous review indicating that the average length of AMP sequences is 10 to 60 amino acids (Huan et al., 2020; Huang and Li, 2023). The reasoning for C-terminus location for this study; was based upon previous research involving lysin PlyPa01 (Heselpoth et al., 2022). PlyPa01 is comprised of 143 amino acids in total length, and the muramidase domain was located between amino acids 7 – 140 inclusively, and its putative membrane-acting C-terminal segment was located between amino acids 103-143. The C-terminal end had a significantly higher theoretical isoelectric point then the N-terminal segment (defined as between the first 1-102 amino acid sequence). Since the isoelectric point is defined as the pH where the net charge of a protein equals zero (Tokmakov et al., 2021), this would indicate that by comparison that the C-terminal end would be more positive due to the presence of more positively charged amino acids.

Therefore our analysis of amino acid features was built upon previously mentioned research on the importance of peptides that disrupt the LPS lipid A and the outer membrane. We utilized this concept by requiring the AMP-like sequence to be found within the 50 amino acid C-terminal end of the lysin protein sequence. Here we propose that the AMP-like domain function, combined with the characterized lysin enzymatic domain function, could not only disrupt the outer membrane of Gram-negative bacterium *Pseudomonas aeruginosa,* but it could also promote the

lysin enzymatic function to further destroy the bacteria through peptidoglycan degradation (if required).

Host bacterium lysin selection

P. aeruginosa was selected for the Gram-negative bacterium host and *S. aureus* was used as a Gram-positive bacterium for comparison lysin mining. *S. aureus* was selected to test our hypothesis that AMP-like sequences would only be present in lysins that destroyed Gram-negative bacteria and not present in lysins aimed at destroying Gram-positive bacteria. Both pathogens are also good choices due to their high levels of antibiotic resistance (Lister, et al., 2009; Chambers and DeLeo, 2009).

Data collection from the PhaLP lysin database

For this specific study, data mining was used within the PhaLP.org database (Criel et al., 2021). In the creation of this comprehensive database, all known and experimentally studied lysins were manually curated, before performing extensive blast searches of published genomes to identify additional phage lysins. In total, there are 16095 entries of potential lysin sequences in the PhaLP database. We queried the PhaLP database to extract all lysins specifically from *P. aeruginosa* and *S. aureus*. In addition to the lysin amino sequence data, we also extracted meta data that included the UniProt accession number, UniProt name, lysin type (VAL or endolysin), protein length, isoelectric point, hydrophobicity values, aromaticity values, and the host phage name. The PhaLP database retrieved phage lytic protein sequence information from various databases including: UniProt, UniParc, NCBI taxonomy, Virus-Host DB, InterPro, GenBank, QuickGO, ExPASy ENZYME database, PDB and PubMed (Criel et al., 2021).

Data maintenance

This lysin sequence data was stored within an excel spreadsheet along with other analysis. There were no confidentiality considerations with this study because experiments with humans were not conducted, and there wasn't any information accessed from studies with specific human subject data. The PhaLP.org database is a publicly shared database of microbial biological data in a specific format that is open to the public, and therefore to any researcher.

Determination of potential lysins with proposed AMP-like features

The database was searched for duplicate lysin sequences, which were defined as any lysin with a minimum of one amino acid difference. All duplicates were removed for all subsequent analysis for both *P. aeruginosa* and *S. aureus*. Next, we extracted the C-terminal 50 amino acid ends of each lysin for both *P. aeruginosa* and *S. aureus*, and then evaluated the quantity of charged amino acids present (arginine, histidine, lysine, aspartic acid and glutamic acid) as well as the overall net charge from the presence of these amino acids, in the 50 amino acid coterminal ends of the lysins. Then we extracted the hydrophobic amino acids present (valine, leucine, isoleucine, alanine, proline, methionine, phenylalanine, tryptophan and cysteine) and evaluated the group of hydrophobic amino acids present for content and frequency. The C-terminal length of 50 amino acids was decided upon due to the aforementioned research for the intent to maximize the chances of finding antimicrobial peptide-like features (Heselpoth et al., 2022; Vazquez et al., 2022; Huan et al., 2020).

The sum of all charged amino acids or hydrophobic amino acids present within each 50 amino acid co-terminal ends of the lysin sequence were calculated as well as respective percentages. From there, we tested for normality of the variances between the two lysin data sets

using the Kolmogorov-Smirnov test using Graph Pad by Prism, with our p-value being set to $p<0.05$. Our obtained D value was 0.3809, and therefore in consideration with our set p-value of $p<0.05$, we determined that the variances of the lysin dataset samples did not follow a normal distribution, and therefore rejected the null hypothesis that the variances from the lysin dataset samples were normally distributed. We then proceeded with using the two-tailed Welch's t-test using Graph Pad by Prism, to examine the differences of the means of the lysins dataset samples for overall net charge. The two-tailed Welch's t-test is a statistical test used to compare the means of two independent groups specifically designed for situations where the two groups are likely to have unequal variances (different standard deviations) as well as having larger sample sizes. The sample sizes of each lysin group of *Pseudomonas aeruginosa* and *Staphylococcus aureus* had different population sizes (as *P. aeruginosa* had 167 lysins and *S. aureus* had 343 lysins). We also performed the Welch's t-test for the differences of the means for hydrophobicity for both groups of lysins. This was done in order to determine whether or not the means were significantly different using a p value of $p<0.05$. Summary graphs were then created within excel to display the results of the findings and can be viewed below in the results section. The presence and frequency of arginine and tryptophan quantities together within their respective 50 amino acid C-terminal lysin sequence were also closely examined. We then compared these various protein qualities between *P. aeruginosa and S. aureus*. Enzymatic type was also evaluated as well as secondary structure of the resulted candidates. Phylogenetic placement of lysins belonging to each dataset was also observed and findings recorded.

Results

i) Identification and Phylogenetic Relationship of all Predicted Lysins from *Pseudomonas Aeruginosa* and *Staphylococcus aureus*

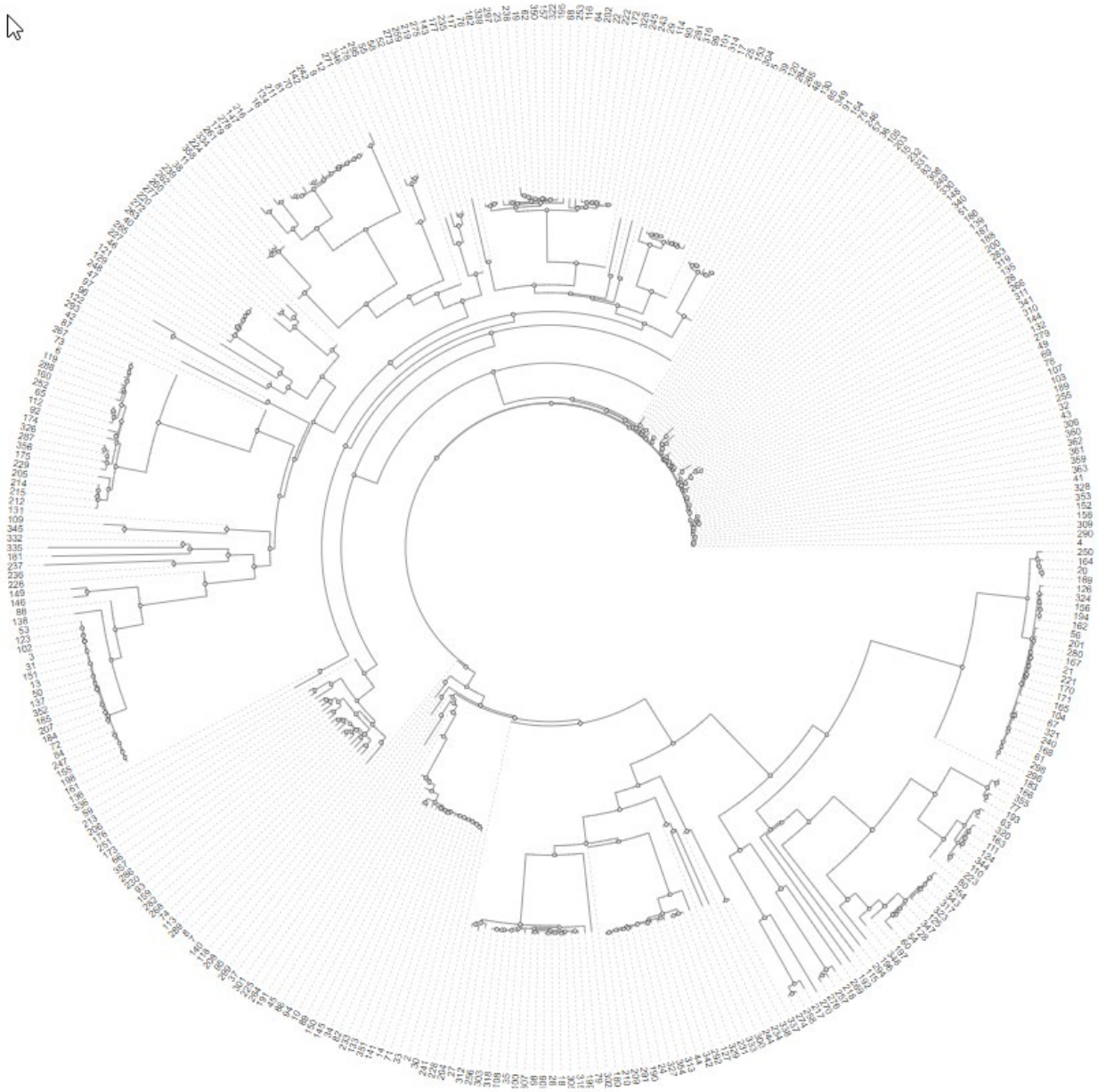
Our objective was to investigate whether lysins that target *P. aeruginosa* possess novel AMP-like features that disrupt the outer membrane, as this would support their development as future enzybiotics. These features would include higher net charge values, higher hydrophobicity as well as increased amounts of tryptophan and arginine occurring together, alongside their relevant Uniprot lysin enzymatic function type.

Once the lysin data was acquired from the PhaLP database with its associated metadata, the duplicates were then removed, and the study accession numbers were applied for each organism, using the > sign to precede the number, starting with "1" for the first lysin and then onwards. A total of 1419 of *P. aeruginosa* lysins were initially identified, which was then reduced and numbered from 1-167 as unique lysins (study accessions: >1 to >167). An initial total 4412 of *S. aureus* lysins were identified and then reduced to 343 unique lysins, numbered 1-343 (study accessions: >1 to >343).

Radial phylogenetic trees were then constructed using the Clustal Omega software (Madeira, et al., 2024) to show the clustering of the lysin datasets for both *P. aeruginosa* and *S. aureus* as well as to ascertain relatedness and ancestral lineage, and if some specific properties, such as enzymatic clustering were more frequent in specific end clusters. These phylogenetic trees for both *P. aeruginosa* and *S. aureus* can be seen below in both Figures 7 and 8 respectively.

Figure 7. Phylogenetic Tree of Unique *Pseudomonas aeruginosa* Lysins

Figure 8. Phylogenetic Tree of Unique *Staphylococcus aureus* Lysins

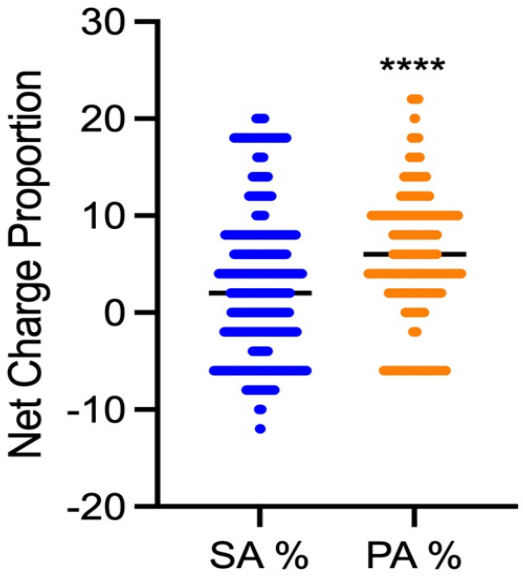


Antimicrobial peptides are rich in hydrophobic and positively charged amino acid residues, which are required for their antimicrobial activity. According to our hypothesis, it was predicted that the C-terminus of that target Gram-negative lysins will have more features for antimicrobial peptide sequences than lysins that target Gram-positive bacteria.

ii) Net Charge Proportion for *Pseudomonas aeruginosa* Lysins vs *Staphylococcus aureus* Lysins

Since antimicrobial peptides most often have a net positive charge to help them interact with the negatively charged outer membrane of bacteria, overall net charge proportion within the 50 amino acid coterminal end was also analyzed for *P. aeruginosa* lysin and *S. aureus* lysin datasets in this paper. This analysis included the quantifying the proportion of positive and negative charged amino acids present in the 50 amino acid C-terminus for both *P. aeruginosa* lysins and *S. aureus* lysin datasets. These amino acids included: arginine, histidine, lysine, aspartic acid and glutamic acid. A graph was produced with charged proportion values for each set of data from both *P. aeruginosa* lysins and *S. aureus* lysins and the results can be seen below in Figure 9. The means for the datasets were then compared using a Welch's t-test.

Figure 9. Net Charge Proportion Percentage for *Pseudomonas aeruginosa* lysins vs *Staphylococcus aureus* lysins



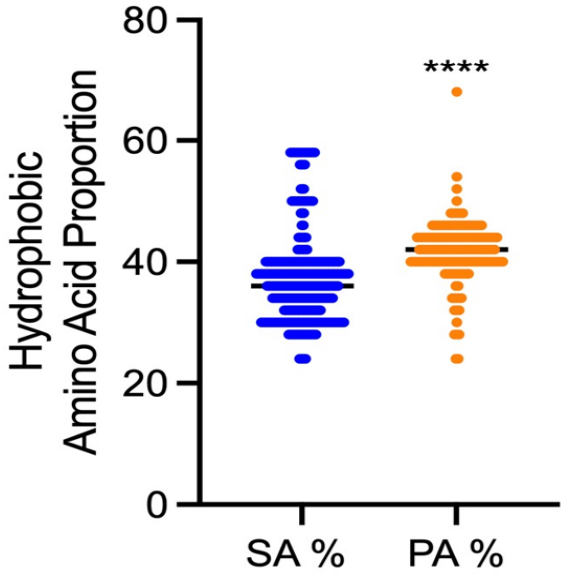
The p value obtained from the Welch's t-test was $p<0.0001$, which was less than our p value of $p<0.05$, and therefore indicates significant statistical difference between the two lysin dataset means. This would indicate that this antimicrobial peptide feature seems to increase in Gram-negative lysins as predicted.

iii) Hydrophobicity Comparison Between Gram-negative *Pseudomonas aeruginosa* Lysins and Gram-positive *Staphylococcus aureus* Lysins

We produced a hydrophobicity graph to observe the differences between Gram-negative *P. aeruginosa* and Gram-positive *S. aureus* hydrophobicity values within the 50 amino acid coterminal end of the lysins for each dataset for *P. aeruginosa* and *S. aureus*. As shown below in Figure 10, the hydrophobicity values for *P. aeruginosa* range from 24% - 68%, which is similar to the same values from *S. aureus*, which range from 24% to 58%. However, the mean value of

hydrophobicity was observed to be higher for *P. aeruginosa* lysin dataset at 41.7%, compared to 37.3% for the *S. aureus* lysin dataset. This suggests that on average, there is a higher percentage of hydrophobic amino acids within the C-terminal end of Gram-negative lysins, which is consistent with our hypothesis.

Figure 10. Hydrophobicity for *Pseudomonas aeruginosa* lysins vs *Staphylococcus aureus* lysins

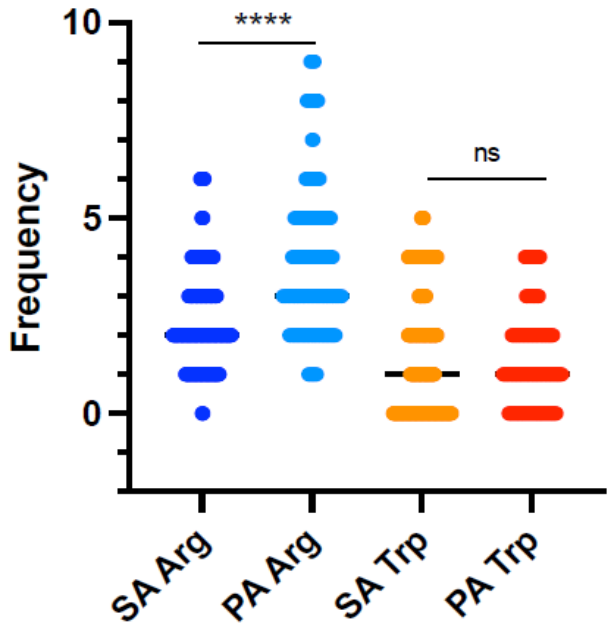


Our results also demonstrated that not only was the hydrophobicity for lysins that target *P. aeruginosa* greater than *S. aureus,* our Welch's two tailed test performed for the hydrophobicity proportions confirmed that the difference was indeed significant, with a p-value of <0.0001.

iv) Examining the Relationship with Tryptophan and Arginine in the C-terminus of Lysins for Gram-negative *Pseudomonas aeruginosa* and Lysins for Gram-positive *Staphylococcus aureus*

It is known that arginine has demonstrated enhancement of antimicrobial ability due to its' guanidinium side chain (Chan et al., 2006). The guanidinium side chain's ability to form bidentate hydrogen bonds with the lipid A core of the Gram-negative outer membrane to its' phosphate groups provides the necessary requirements of conferring peptide charge as an amino acid and creating hydrogen bond interactions with the Gram-negative *P. aeruginosa's* membrane's anionic environment. Therefore these amino acids were of primary interest due to these properties, as they may enhance antimicrobial ability of lysins. Given the different membrane structures, we hypothesized that this antimicrobial peptide feature would be more prominent in *P. aeruginosa* compered to *S. aureus*. It was observed in Figure 11 that there was a higher amounts of arginine present in *P. aeruginosa* than for *S. aureus* in the 50 amino acid C-terminus but no statistical difference in the amounts of tryptophan present.

Figure 11. Arginine to Tryptophan Frequencies for *Pseudomonas aeruginosa* lysins and *Staphylococcus aureus* lysins



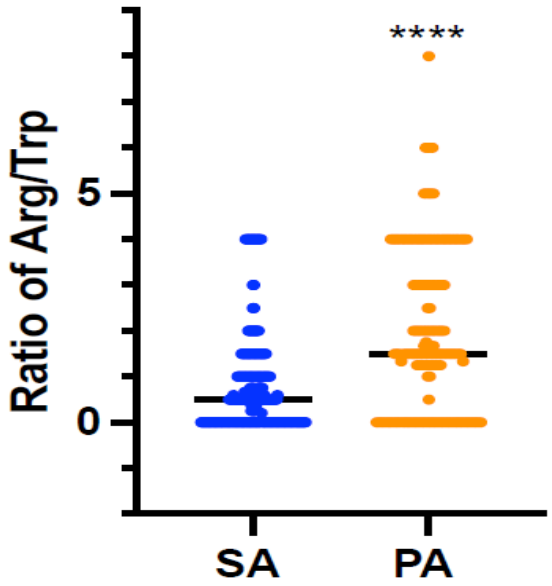
Also, in the *P. aeruginosa* lysin dataset there was frequently an increase in tryptophan that was proportionate with an increase in arginine, which was not observed in the *S. aureus* lysin dataset. From our extracted dataset for *Pseudomonas aeruginosa* lysins where we observed certain lysins having the highest amount of both arginine and tryptophan present together in quantities of five arginines to four tryptophans per lysin in the 50 amino acid coterminal end, and these lysins were as follows: >3, >26, >40, >42, >51, >67, >123, >132, >141, >146, and >149 and can be summarized below in Table 2.

Table 2. Arginine and Tryptophan Quantities Present in 50 Amino Acid Coterminal End of the eleven Selected Lysins from the *Pseudomonas aeruginosa* Dataset

Lysin	Arginine Quantity Present	Tryptophan Quantity Present
>3	5	4
>26	5	4
>40	5	4
>42	5	4
>51	5	4
>67	5	4
>123	5	4
>132	5	4
>141	5	4
>146	5	4
>149	5	4

In addition to the quantities present we also observed an increase in the overall ratio of arginine and tryptophan present for the *Pseudomonas aeruginosa* lysin dataset when compared to the *Staphylococcus aureus* lysin dataset in Figure 12. This further supports our hypothesis in that antimicrobial peptide features are enhanced in lysins that target Gram-negative bacteria.

Figure 12. Arginine to Tryptophan Ratios for *Pseudomonas aeruginosa* and *Staphylococcus aureus* Lysin C-terminal 50 Amino Acid Regions



v) Examining the Relationship with Hydrophobicity with Tryptophan and Arginine in the 50 Amino Acid C-terminus of Lysins for Gram-negative *Pseudomonas aeruginosa*

After observing an increased presence of arginine to tryptophan and a higher arginine to tryptophan ratio in *P. aeruginosa* lysins, further analysis was then performed to assess hydrophobicity within the lysins for *P. aeruginosa* that had the aforementioned presence of increased arginine and tryptophan quantities present (>3, >26, >40, >42, >51, >67, >123, >132,

>141, >146, and >149). Our findings indicated that this specific group of lysins all shared a hydrophobicity value of 46%, which was indeed higher than the mean average for the *P. aeruginosa* lysin dataset of 41.7%.

These eleven priority lysin sequences with a higher hydrophobic proportion of 46% also were deemed cationic, as the net charge was identified for each lysin in this group as +2, which is summarized below in Table 3.

Table 3. Hydrophobic Proportion and Net Charge Present in the 50 Amino Acid C-terminal end of the eleven Selected Lysins of the *Pseudomonas aeruginosa* Dataset

Lysin	Hydrophobic Proportion %	Net Charge
>3	46%	+2
>26	46%	+2
>40	46%	+2
>42	46%	+2
>51	46%	+2
>67	46%	+2
>123	46%	+2
>132	46%	+2
>141	46%	+2
>146	46%	+2
>149	46%	+2

vi) Selection of Most Suitable Candidate Lysins with Antimicrobial Peptide Properties within the 50 Amino Acid C-terminus of *Pseudomonas aeruginosa*

Our analysis suggests that the C-terminal end of lysins from *P. aeruginosa* have qualities of antimicrobial peptides. We reasoned that lysins in Gram-negative bacteria have evolved to include AMP-like properties in the C-terminal ends which would promote bacterial killing from the exterior surface in addition to the lysin enzymatic role of degrading peptidoglycan, after crossing the inner membrane during phage release.

Lysin enzymatic classification was identified in PhaLP via Uniprot classification system and this classification parameter had been selected upon data retrieval from PhaLP. Upon further analysis on our existing 11 candidate lysins, it was decided that only lysins that had a specific lysin categorization type (as defined previously in this paper) were prioritized. This further refined our candidates down to eight potentials. In our case all eight candidates possessed the Uniprot classification as "lysozyme" and are as follows: >3, >40, >42, >51, >67, >123, >132, and >146. These eight candidates are shown below in Table 4 and are further characterized with their Uniprot accession number, host species, phage name and enzyme classification type.

Table 4. Proposed candidate lysins for *Pseudomonas aeruginosa*

Lysin (Study Accession Number)	Uniprot Accession Number	Host Species	Phage Name	Enzyme Classification Type
>3	H8ZM03	*Pseudomonas aeruginosa*	*Pseudomonas* phage MR299-2	Lysozyme
>40	W0XA98	*Pseudomonas aeruginosa*	*Pseudomonas* phage TL	Lysozyme

>42	K8DWG8	*Pseudomonas aeruginosa*	*Pseudomonas* phage vB_PaeP_p2-10_Or1	Lysozyme
>51	V5R527	*Pseudomonas aeruginosa*	*Pseudomonas* phage phiIBB-PAA2	Lysozyme
>67	A0A386K578	*Pseudomonas aeruginosa*	*Pseudomonas* phage phiPA01_302	Lysozyme
>123	A0A386K5T5	*Pseudomonas aeruginosa*	*Pseudomonas* phage phiPA01_EW	Lysozyme
>132	A0A3G1L343	*Pseudomonas aeruginosa*	*Pseudomonas* phage Delta	Lysozyme
>146	A0A5Q5ANW9	*Pseudomonas aeruginosa*	*Pseudomonas* virus Pa222	Lysozyme

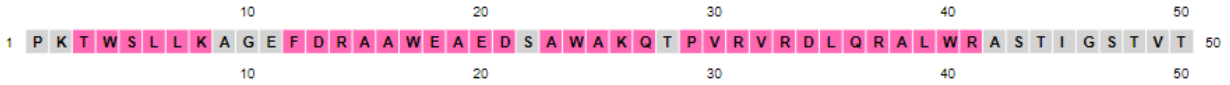
vii) Proposed Secondary Structure Findings of C-terminal sequences from the list of Proposed Candidate Lysins for *Pseudomonas aeruginosa*

As already stated, AMP membrane disruption can occur through differing proposed models and these proposed models outline the application of helix configuration that assists with AMP outer-membrane permeation. In order to determine if the 50 amino acid C-terminus sequence could encode sequences that form final structures that resembled potential antimicrobial peptides, we then examined the secondary structures for each candidate using the online software: PSIPRED (Buchan, 2024).

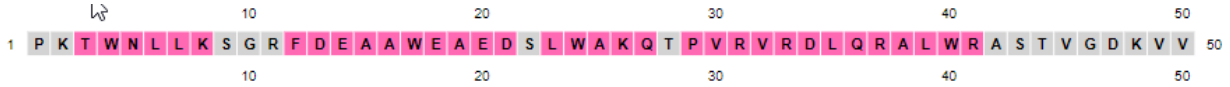
As can be seen below in Figure 13, each candidate possessed helical sequences within the lysin's 50 amino acid C-terminus. This is further evidence that these lysins sequences may possess embedded AMP-like sequences that have potential outer membrane disrupting activity.

Figure 13. Predicted Secondary Structure Findings for Potential Candidate Lysins for *Pseudomonas aeruginosa*

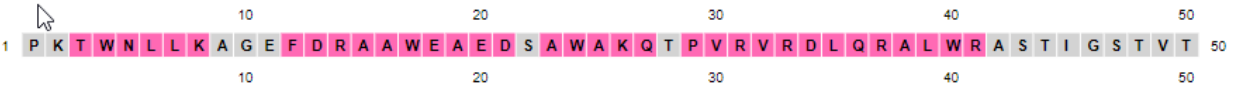
Study Numeric >3 Assigned to Accession: H8ZM03 ***Pseudomonas*** **phage**



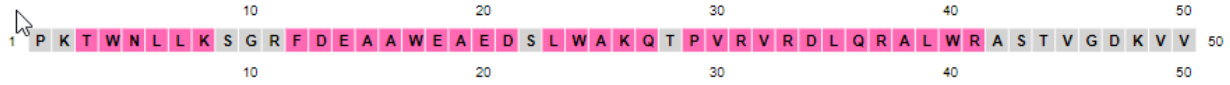
Study Numeric >40 Assigned to Accession: W0XA98 ***Pseudomonas*** **phage**



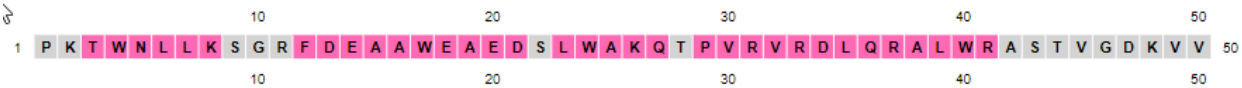
Study Numeric >42 Assigned to Accession: K8DWG8 ***Pseudomonas*** **phage**



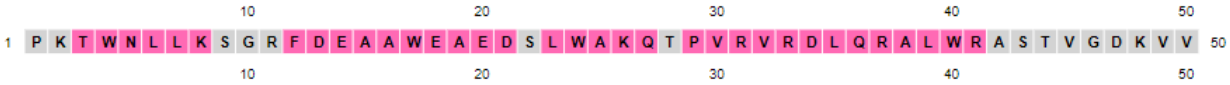
Study Numeric > 51 Assigned to Accession: V5R527 ***Pseudomonas*** **phage**



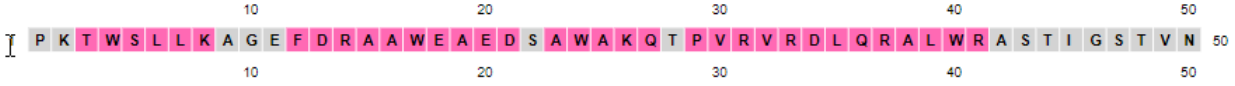
Study Numeric > 67 Assigned to Accession: A0A386K578 ***Pseudomonas*** **phage**



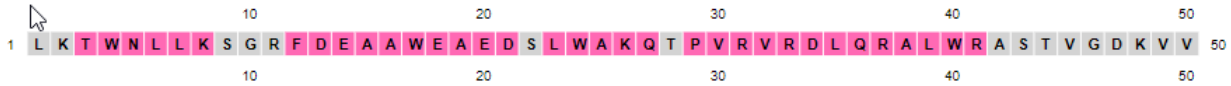
Study Numeric > 123 Assigned to Accession: A0A386K5T5 ***Pseudomonas*** **phage**



Study Numeric > 132 Assigned to Accession: A0A3G1L343 ***Pseudomonas*** **phage**



Study Numeric > 146 Assigned to Accession: A0A5Q5ANW9 ***Pseudomonas*** **phage**

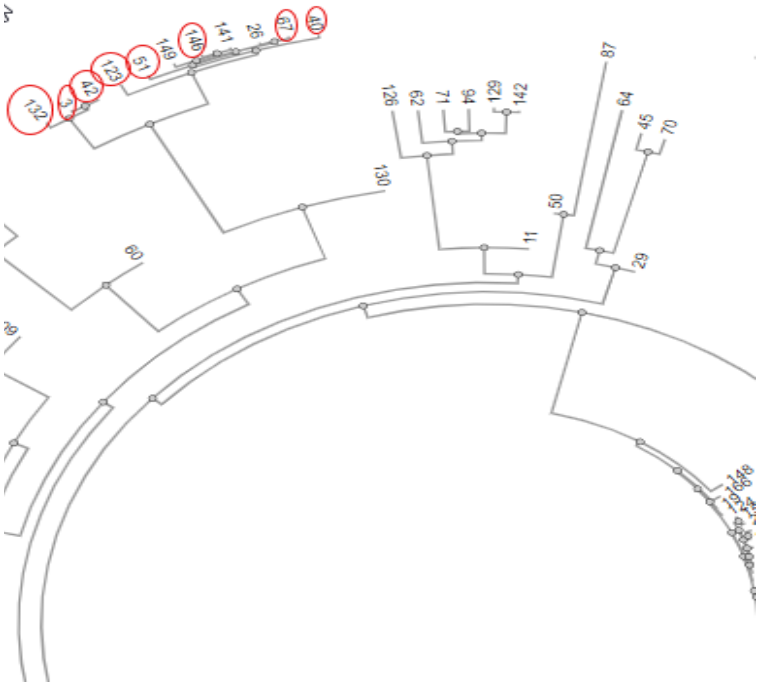


PSIPRED Workbench Legend:

viii) Further Findings of Eight Candidate Lysins Based off of Location within the Phylogenetic Tree and Sequence Similarity of *Pseudomonas aeruginosa* Unique Lysins

Upon confirmation of identical placement of helical structures within our already curated list of eight potential lysins, it was observed below in Figure 14 that the candidate sequences were quite similar, as they were found to be clustered together in close proximity with one another within our phylogenetic tree generated from Figure 7. This finding, in combination with their shared hydrophobicity value of 46% and net charge value of 2+, would strongly indicate that this specific cluster was representative of the same lysin.

Figure 14. Cluster Location of eight Candidate Lysin Sequences within the Phylogenetic Tree of *Pseudomonas aeruginosa* Unique Lysins



Due to this finding of high sequence similarity, it was decided to refine the list of eight candidates to two candidates: >40 (UniProt accession: W0XA98) and >132 (UniProt accession: A0A3G1L343). These two candidates exhibited the greatest difference amongst the eight lysins within our list in terms of sequence similarity, as well as demonstrating identical placement of helical predicted structure within the 50 amino acid C-terminus. They also exhibited the greatest distance from each other within the clustering of all previous eight candidates. To validate our findings, future testing would be necessary to indicate whether or not these factors of difference affected their efficacy (should efficacy was demonstrated with either candidate in vitro).

After this observation, we also decided to include additional candidates from other clusters, as we observed that there were relationships within the clusters involving net charge and

hydrophobicity of lysin 50 amino acid C-terminal ends. Many clusters shared the same net charge, as well as shared the same hydrophobic proportion percentages. Therefore, our additional candidate selection included lysins from clusters with net charges ranging from +2 to +9, due to previous literature that had stated most AMPs are within this net charge range (Haney and Hancock, 2013). Also, our selected net charge clusters had hydrophobic proportion values that were averaged above the mean for the *Pseudomonas aeruginosa* dataset of 41.7%. We then selected one lysin per cluster respective to these properties (excluding the cluster shown in Figure 14). These additional candidates lysins: >74, >104, >114, >116, >124, and >158, are further summarized with their cluster net charge and hydrophobicity proportion percentages shown in Table 5 below.

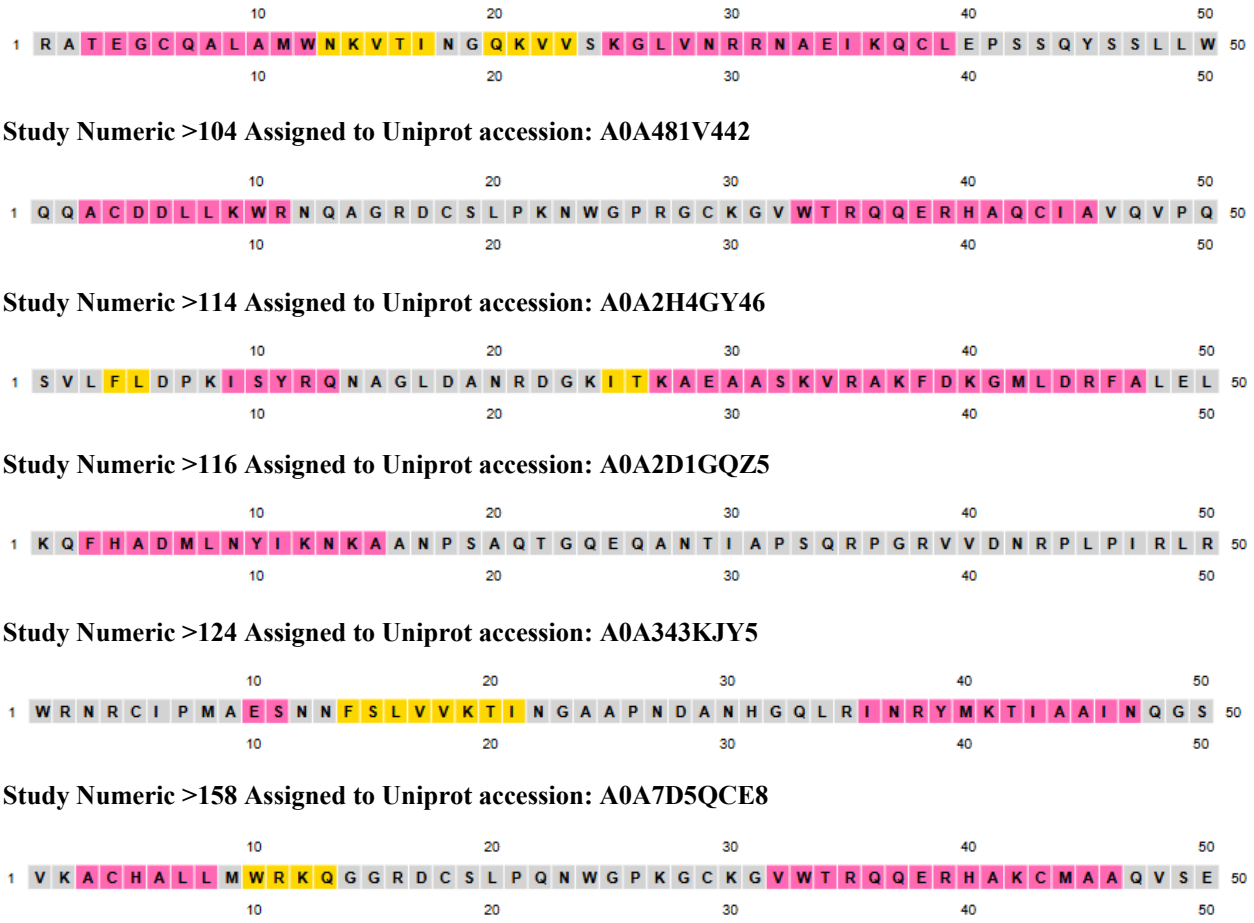
Table 5. Additional Proposed Candidate Lysins for *Pseudomonas aeruginosa*

Lysin (Study Accession Number)	Uniprot Accession Number	Host Species	Net Charge Value	Hydrophobicity Proportion %	Enzyme Classification Type
>74	A0A0A1IVZ0	*Pseudomonas aeruginosa*	+4	42%	Lysozyme
>104	A0A481V442	*Pseudomonas aeruginosa*	+5	42%	Lysozyme
>114	A0A2H4GY46	*Pseudomonas aeruginosa*	+3	44%	(putative)Lytic transglycolase
>116	A0A2D1GQZ5	*Pseudomonas aeruginosa*	+6	42%	(putative)Lytic transglycolase
>124	A0A343KJY5	*Pseudomonas aeruginosa*	+5	44%	Lysozyme
>158	A0A7D5QCE8	*Pseudomonas aeruginosa*	+8	44%	Lysozyme

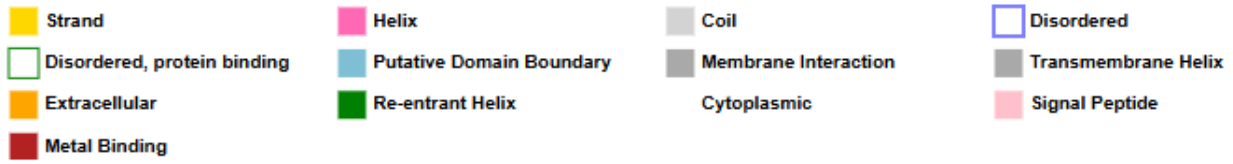
We also wanted to determine if the 50 amino acid C-terminus sequence could encode for final structures that would potentially resemble antimicrobial peptides, and therefore we utilized the online software: PSIPRED (Buchan, 2024).

As shown in Figure 15, each of the additional candidates possessed helical sequences within the C-terminus. This is further supports that these additional lysin sequences may possess embedded AMP-like sequences which have potential outer membrane disrupting activity.

Figure 15. Predicted Secondary Structure Findings for Additional Potential Candidate Lysins for *Pseudomonas aeruginosa*



PSIPRED Workbench Legend:



Discussion

Our work provides a new perspective on AMP-like domains present in some lysins that target Gram-negative *Pseudomonas aeruginosa*. Previous studies have identified antimicrobial sequences within the C-terminal end of phage lysins that target *Pseudomonas aeruginosa*, and that synthetic peptides derived from these sequences had antimicrobial activity. Here we are expanding the search for AMP-like domains in all phage lysins from Gram-negative *Pseudomonas aeruginosa* and Gram-positive *Staphylococcus aureus*. We were specifically interested with their net charge positive values, hydrophobic proportion quantities, as well as further examining the presence of higher arginine to tryptophan ratios present and whether these specific amino acids may provide greater antimicrobial peptide-like properties in lysins that target Gram-negative *Pseudomonas aeruginosa*.

To test our hypothesis, we utilized a recently published PhaLP database, that comprehensively predicted and classified all bacterial lysins. Our aim was to identify whether lysins retrieved from *P. aeruginosa* and *S. aureus* genomes possess the previously outlined AMP-like characteristics within their 50 amino acid coterminal ends.

We observed that through statistical testing that *P. aeruginosa* lysins had higher net charge values as well as higher hydrophobicity values and higher arginine and tryptophan ratios than *S.*

aureus lysins. We therefore concluded that from this data that *P. aeruginosa* demonstrated a greater likelihood of possessing potential AMP-like characteristics from either organism, and therefore focused on identifying specific priority lysins from *P. aeruginosa.*

We initially discovered a group of lysins that were closely phylogenetically related that not only shared the same overall net positive charge value and hydrophobicity percentage proportion percentages, but also specific amino acid composition characteristics. This satisfied the aforementioned criteria regarding successful AMPs possessing both cationic and hydrophobic amino acid residues in high proportions (Huan et al., 2014). These proteins also boasted the predicted presence of helical sequences as seen in Figure 13. Helical sequences in particular have demonstrated success in previous studies for antimicrobial activity (Islam et al., 2023; Yang et al., 2018). Due to high phylogenetic relatedness and sequence similarity within this single cluster, it was then decided to prioritize two primary lysins from our initially discovered group: study accession >40 (UniProt accession: W0XA98) and study accession >132 (UniProt accession: A0A3G1L343). These lysins manifested the greatest sequence difference by a total of nine amino acids out of the prior eight lysins selected. They also were the furthest apart distance-wise within the cluster of their phylogenetic tree of *P. aeruginosa* unique lysins (Figure 7). These two lysins were the primary selections for future work to determine the presence of successful lysin function.

This was followed by our phylogenetic tree analysis that assessed the net charge and hydrophobicity of each cluster, as well as also having formal Uniprot lysin enzyme classifications. From this perspective we selected six additional candidates: >74 (UniProt accession: A0A0A1IVZ0), >104 (UniProt accession: A0A481V442), >114 (UniProt accession: A0A2H4GY46), >116 (UniProt accession: A0A2D1GQZ5), >124 (UniProt accession:

A0A343KJY5), and >158 (UniProt accession: A0A7D5QCE8) for future studies, which could be assessed using conventional minimum inhibitory concentration (MIC) testing to measure antimicrobial activity as well as assays that measure specific outer membrane damage.

Conclusion

The future confirmation of improved antimicrobial activity among the lysins prioritized within this bioinformatic study would provide researchers with a new arsenal of lysin candidates to be pursued as novel enzybiotic antimicrobials that target *P. aeruginosa*, as well as targeting other Gram-negative species.

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Appendix A: WHO Priority Pathogens List for Antibiotic Resistant Pathogens

Priority 1: CRITICAL

- *Acinetobacter baumannii*, carbapenem-resistant – **GRAM NEGATIVE**
- *Pseudomonas aeruginosa*, carbapenem-resistant – **GRAM NEGATIVE**
- *Enterobacteriaceae*, carbapenem-resistant, ESBL-producing – **GRAM NEGATIVE**

Priority 2: HIGH

- *Enterococcus faecium*, vancomycin-resistant – **GRAM POSITIVE**
- *Staphylococcus aureus*, methicillin-resistant, vancomycin-intermediate and resistant - **GRAM POSITIVE**
- *Helicobacter pylori*, clarithromycin-resistant - **GRAM NEGATIVE**
- *Campylobacter* spp., fluoroquinolone-resistant - **GRAM NEGATIVE**
- *Salmonellae*, fluoroquinolone-resistant - **GRAM NEGATIVE**
- *Neisseria gonorrhoeae*, cephalosporin-resistant, fluoroquinolone-resistant - **GRAM NEGATIVE**

Priority 3: MEDIUM

- *Streptococcus pneumoniae*, penicillin-non-susceptible - **GRAM POSITIVE**
- *Haemophilus influenzae*, ampicillin-resistant - **GRAM NEGATIVE**
- *Shigella* spp., fluoroquinolone-resistant - **GRAM NEGATIVE**

Appendix B: Table 3. Welch's unpaired T-test for hydrophobicity means between *Pseudomonas aeruginosa* lysins and *Staphylococcus aureus* lysins

P value	**<0.0001**
P value summary	********
Significantly different (P<0.05)?	**Yes**
One- or two-tailed P value	**Two-tailed**
Welch-corrected t, df	**t=8.203, df=458.9**
Mean of *S. aureus*	**37.25**
Mean of *P. aeruginosa*	**41.72**
Different between means (B-A) +/- SEM	**4.479 +/- 0.5460**
95% confidence interval	**3.406 to 5.552**
R squared (eta squared)	**0.1279**
F, DFn, DFd	**2.222, 362, 166**
P value	**<0.0001**
P value summary	********
Significantly different (P<0.05)?	**Yes**
Sample size *S. aureus*	**363**
Sample size *P. aeruginosa*	**167**

Appendix C: Table 4. Welch's T-test for net charge means between *Pseudomonas aeruginosa* lysins and *Staphylococcus aureus* lysins

P value	**<0.0001**
P value summary	********
Significantly different (P<0.05)?	**Yes**
One- or two-tailed P value	**Two-tailed**
Welch-corrected t, df	**T=3.992, df=371.3**
(A) Mean of *S. aureus*	**3.229**
(B) Mean of *P. aeruginosa*	**5.784**
Different between means (B-A) +/- SEM	**2.556 +/- 0.6403**
95% confidence interval	**1.297 to 3.815**
R squared (eta squared)	**0.04114**
F, DFn, DFd	**1.355, 362, 166**
P value	**0.0259**
P value summary	*****
Significantly different (P<0.05)?	**Yes**
Sample size *S. aureus*	**363**
Sample size *P. aeruginosa*	**167**