BREAKING INTERINSIC ANTIBIOTIC RESSISTANCE

OUTER MEMBRANE-PERMIABLIZING PEPTIDES FROM INNATE BACTERIAL PROTEOMES

A DISSERTATION
SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE
OF

MATERS OF SCIENCE
IN
BIOTECHNOLOGY

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CANDIDATE'S DECLARATION

I, Pragya Kamal, Roll No. 2K20/MSCBIO/21 M.Sc. Biotechnology student declares that the project report titled "Breaking the intrinsic antibiotic resistance -Outer membrane permeabilizing peptides from innate bacterial proteomes" which has been submitted to the Department of Biotechnology, Delhi Technological University, New Delhi, is original and has not taken from any of the sources without proper citation. This work has not previously formed the basis for the award of any Degree, Diploma Associateship, Fellowship or other similar title or recognition.

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Date:

PRAGYA KAMAL
2K20/MSCBIO/21

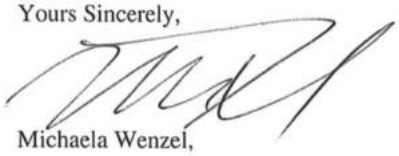
CERTIFICATE

I hereby certify that the dissertation project titled **"Breaking the intrinsic antibiotic resistance -Outer membrane permeabilizing peptides from innate bacterial proteomes"** which is submitted by Pragya Kamal, 2K20/MSCBIO/21, Department of Biotechnology, Delhi Technological University, Delhi in partial fulfilment of requirement for the award of the degree of Master of Science, is a record of work carried out by the student under my supervision. To the best of my knowledge this work has not been submitted in part of any Degree or Diploma to this University or elsewhere.

Date: 02-05-2022

Place: Sweden

Yours Sincerely,



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I hereby certify that the dissertation project titled **"Breaking the intrinsic antibiotic resistance -Outer membrane permeabilizing peptides from innate bacterial proteomes"** which is submitted by Pragya Kamal, 2K20/MSCBIO/21, Department of Biotechnology, Delhi Technological University, Delhi in partial fulfilment of requirement for the award of the degree of Master of Science, is a record of work carried out by the student under the supervision of Dr. Michieala Wenzel, Chalmers University of Technology, Sweden. I trust the statement of Dr. Wenzel as she states that this work has not been submitted in part of any Degree or Diploma to this University or elsewhere.

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Abstract

Intrinsic antibiotic resistance is the innate ability of bacteria to resist antimicrobial agents. The most defining structure that confers intrinsic antibiotic resistance, is the outer membrane of Gram-negative bacteria. It is a highly impermeable barrier that impedes various antibiotics from reaching their targets. A lot of research focuses on finding and developing novel drug candidates with increased antibacterial activity, but less research has been done on selective outer membrane permeabilization compounds. Such compounds could render Gram-negative bacteria susceptible to almost all known antimicrobials. It has been shown that the bacterial proteome is host to proteins containing a membrane-anchoring motif, which exerts selectivity towards the outer membrane.

The aim of this Master thesis is to investigate the outer membrane permeabilizing properties of peptides derived from membrane-binding domains of innate peptides. This is realized through development and optimization of assays to assess the peptides outer and inner membrane activity against *Mycobacterium smegmatis* In addition, antibacterial activity was tested against both *E. coli.*

Here I show that 16 out of 18 innate peptides derived from *M. smegmatis* and *E.coli* are active against the outer membrane of *M. smegmatis*. Expect peptides SepF11 and SepF14 all the other peptides shown outer membrane permeabilization in Mycobacterium smegmatis which is a very interesting finding and can be further used to solve the the problem of membrane permeabilising by antibiotics and may be used to solve the antibiotic resistance exist in MDR strains of *Mycobacterium tuberculosis*.

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Abbreviations

AA=Amino acids

AMPs=Antimicrobial peptides

DiSC3(5)= 3,3'-Dipropylthiadicarbocyanine Iodide

DMSO= Dimethyl sulfoxide

HEPES=4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IPTG= Isopropyl β-D1-thiogalactopyranoside

LB=Luria-Bertani

LPS=Lipopolysaccharide

MIC=Minimal inhibitory concentration

MHB=Mueller-Hinton broth

NPN=*N*-phenyl-1-naphtylamine

ONPG=2-Nitrophenyl β-D-galactopyranoside

PolB=Polymyxin B

PMBN=Polymyxin B nonapeptide

PBS=Phosphate buffered saline

Chapter 1: Introduction

Antibiotic-resistant bacteria pose a global health problem with estimates of 700 000 deaths per year globally (WHO, 2019). The rise of antibiotic resistance leads to more infections that can no longer be treated with antibiotics. This problem has mainly emerged because of the misuse of antibiotics, through actions such as unnecessary prescriptions, over-the-counter available antibiotics, overuse in the agriculture and insufficient waste management[1] . However, there are many different ways antibiotic resistance occurs and they can be categorized into two main groups, acquired and intrinsic (Peterson & Kaur, 2018). Acquired antibiotic resistance is caused by the constant exposure of bacteria to antibiotics, which leads to a selection of mutations that increase the resilience of bacterial strains towards certain antibiotics [2]. The mutations can then be passed on to other strains through horizontal gene transfer, thus spreading the antibiotic resistance [2]. Intrinsic antibiotic resistance is not caused by mutations and is instead defined as the innate abilities of bacteria that limit the effects of antimicrobial drugs [3]. The outmost membrane of major Gram-negative bacteria is a defining archetype of intrinsic antibiotic resistance as it inhibits many antibiotics from reaching their targets, which often reside in the inner membrane or the cytoplasm [4]. While there are several antibiotics readily available for treatment of bacterial infections, many of them are rendered useless they are fighting Gram-negative bacteria, because of the presence of outer membrane. This makes Gram-negative bacteria inherently more resilient than Gram-positive bacteria, which lack an outer membrane. It is also one of the main reasons why 9 out of the 12 bacteria on WHOs global priority list are Gram-negative bacteria [5]. Together with the rise in acquired antibiotic resistant, this has led to a decrease in viable treatments for Gram-negative bacterial infections (Sprenger & Fukuda, 2016). There are still antibiotics used against Gram-negative bacteria, but only one class of antibiotics that target the outer membrane are clinically approved, the polymyxins [6]. However, they exhibit severe side effects. Antibiotics are substances that have antibacterial properties and are used to treat bacteria borne infections or diseases. There are two kinds of antibiotics: a) Bacteriocidal that kills the bacteria by interfering with its life dependent mechanisms like preventing the formation of cell wall and b) bacteriostatic that inhibit the bacterial growth by preventing its reproduction. [7] Antibiotics do not work against viruses, however, there are few antibiotics that can work against certain protozoan for example Metronidazole has both antibacterial and antiprotozoal activity. [8] Antibiotics are antibacterial

i.e. they work against bacteria, but these two terms antibiotic and antibacterial are used in very different senses although they seem almost the same. According to the medical vocabulary, substances which are only naturally derived and used in medication are known as antibiotics like penicillin and substances that are synthetically made are called "nonantibiotic antibacterial" or simply antibacterial. Examples of this category are antiseptics and disinfectants which are chemically derived and are not suitable to be used as medicines for humans or animals. However, the basic function of either antibiotic or antibacterial products is to stop bacterial growth or kill the bacteria, but usage of these terms refined during the course of time and are now used to refer two different things. With the introduction of antibiotics a dramatic reduction took place towards infectious diseases, yet new bacterial diseases keep on coming into sight. This, together with the increased usage of existing antibiotics cause the antibiotic resistance which still remains an area of major concern.

1.1 Antimicrobial peptides:

Antibiotics are becoming less and less efficient. Therefore, researching new strategies and developing drugs is very important. Organisms have a natural defense against bacterial infections e.g. antimicrobial peptide (AMP). Almost all organisms express these AMPs. For example, they play a very important role in the innate immune system against infections. They have multiples advantages like a board spectrum activity, rapid action, and difficult development of resistance. Even though these AMPs exist for millions of years, resistance against them is rare. On the contrary, the evolution of microbes also led to a higher diversity of AMPs. This makes AMPs interesting therapeutic candidates. Generally it is accepted that the AMPs target the cell membrane. This means that the AMPs might interact with eukaryotes cells membranes and cause issues of toxicity to host cells. Therefore, many researches are going on to develop the better understanding of relationship of structure and activity present in AMPs. The aim is to understand the mechanism for designing AMPs with a high antimicrobial activity and no cytotoxicity against eukaryotic cells. Below shown the properties of AMPS:

Diversity	Uniqueness	Length	Charge	Amphipathic
AMPs can be found in many species (bacteria, mammals, ...)	Each AMP is different	Short sequence between 12 and 100 amino acids	Almost charged positively +2 to +9 due to basic amino acids	50% hydrophobic residues

There are different sorts of AMPs for example as the peptaibols or the cationic AMP. Melittin, cecropins and magainins are some examples of these cationic AMPs. However, the proprieties of the cecropins and the magainins are less known that the alamethicin [9][13]. These peptides are charged and soluble in aqueous membranes. The hydrophobic part insertsinto the membrane and causes the disordering of the fatty acyl chain packing, membrane narrowing and pore formation. Both, the negative charge of the cell membrane and the strong electrochemical gradient of the bacterial cytoplasmic membrane, contribute to the attraction of cationic AMPs. The cation of the AMPs replaces divalent cations(Mg2+/Ca2+) and disturbs the rigidity of the outer membrane. Indeed, these divalent cations stabilize the lipopolysaccharides (LPS). Once inserted, AMPs can adopt different conformations:

- **The carpet model:** The peptides remain in contact with the lipid head groups during this model. They do not interact with the hydrophobic part of the membrane and there is no channel.

- **The barrel stave:** The peptides are perpendicular to the phospholipids. When a threshold peptide concentration is reached, the peptides form a dynamic pore. Mostly seen with the eukaryotes and cause the hemolysis of erythrocytes.

- **Toroidal-pore:** Peptides are reoriented perpendicularly similar to the barrel-stave. The peptides form an aqueous pore. The difference to the barrel stave is the tilt of lipid molecules, so the membrane is curved inside to form a hole.

1.2 Properties of AMPs

AMPs are defined by the therapeutic index. This value is calculated with the formula MHC/MICThe MHC represents the maximum peptides concentration with non-hemolytic assay, so the score needs to be as high as possible, and the MIC represents the minimum concentration to have an antimicrobial activity which needs to be low [9].
Many parameters can influence the efficacity of AMPs. These parameters can be changed:
- **Length:** At least 22 residues are required to transvers the lipid bilayer and forms a pore. However, not all AMPs form pores. Smaller peptides can still kill bacteria. The length modification can change the therapeutic index for example a 15 residues synthetic peptide

corresponding to the C-terminus of melittin leads to a 5-7 times less antimicrobial activity, but also 300 times less hemolytic activity compared to normal melittin [9].

- Sequence: A study from Tossi et al. (2000) compared the 20 residues of the N-terminal α-helical domain of over 150 natural AMPs and did not find a conserved pattern. For example, the positional residue conservation was very poor excepting for the position 1 and 8 with 70% of Glycine and 50% Lysine, respectively.

- Charge: Increasing the net charge from +4 to +8 can increase the antimicrobial activity and maintaining a low hemolytic activity at the same time [14].

- Helicity: The substitution of D-amino acid in an AMP may be effective to dissociate the antimicrobial activity against cytotoxicity of eukaryotes cells. The function between L-amino acid and these diastereomers are the same in an amphipathic peptide. However, the diastereomer is less toxic [14].

- Hydrophobicity: There is an optimal hydrophobicity window in which high antimicrobial activity can be reached. Outside this window the antimicrobial activity could be inhibited and on the contrary the hemolytic activity could be higher [14].

- Amphipathicity and hydrophobic moment: The hydrophobic moment can affect the antimicrobial activity more than the hydrophobicity.

- Amidation in C-terminal: Amidation not only modifies the net positive charge, but also stabilizes peptide structure and improves antimicrobial activity.

- Disruption of the hydrophobic surface: This change allows the peptide to enter the membrane interface region more effectively and minimize the hemolytic activity (Huang et al.; 2010).Polymyxins work for treating Gram-negative bacteria by permeabilizing or transiently perturbing the outer membrane. They are used only as last resort antibiotics due to their severe side effects, which include neurotoxicity, nephrotoxicity, numbness or burning

sensation in extremities, loss of coordination and hives. The reason they are used at all is due to the spread of antibiotic resistance that makes them the only viable treatment for some bacterial infections [6]. However, resistance against polymyxins is emerging as well [15][13]. Another member of the polymyxins is polymyxin B nonapeptide (PMBN), which permeabilizes the outer membrane and shows no inner membrane activity [16]. It exhibits a strongly decreased antibacterial activity compared to polymyxin B, but is also much less toxic. Polymyxins are cyclic lipopeptides, which is a sub-category of a larger group called antimicrobial peptides (AMPs) that through varying mechanisms affect bacterial membranes and often show antibacterial activity.

AMPs are naturally occurring defense molecules and are part of various organisms' immune responses. AMPs can also be synthetically designed and in total there are over 2000 listed AMPs so far (Wang *et al*., 2016). Some common features of AMPs are that most are comprised of 10-50 amino acid (AA) residues and have a net positive charge [17]. AMPs have diverse sequences that fold into various structures, including β-sheets and α-helices [18]. α-helical peptides are often unstructured in aqueous solution but conform into an amphipathic α-helix in the presence of bacterial membranes. Many AMPs are so-called amphipaths. This term describes molecules that exhibit both hydrophobic and hydrophilic properties. When studying peptides, amphipathicity refers to the alignment of amino acids in a folded peptide that form one hydrophobic side and one hydrophilic side[19] . This property is essential for AMPs to interact with membranes. Such amphipathic peptides are interesting for antibiotic development due to their ability to disrupt the outer membrane. A problem with many AMPs that exhibited outer membrane activity, is that they are not selective and target the inner membrane of Gram-negative bacteria as well as the outer membrane. Consequently, they often show both antibacterial activity and cytotoxicity or *in vivo* toxicity[20]. One way to solve this problem could be to search for AMPs that show a low antibacterial activity while still retaining the ability to permeabilize the outer membrane, such as the polymyxin derivate PMBN. [16]These compounds could be used as antibiotic adjuvants, which can sensitize bacteria without killing them but instead allow access of existing antimicrobial drugs to their target [21].

In the search for novel AMPs, the use of membrane-binding domains from the proteins FtsA and MreB was examined [22]. These are innate proteins to *Escherichia coli*, which are involved in the cell division cycle[23].

All studied peptides derived from FtsA and MreB showed outer membrane permeabilization and all modified peptides showed inner membrane and antibacterial activity as well [24]. The unmodified peptides FtsA10 and MreB9 were disregarded for future development due to their lack of inner membrane and antibacterial activity. However, despite being poor inhibitors of growth, they selectively permeabilized the outer membrane at low micromolar concentrations, making them interesting for developing antibiotic adjuvants. The fact that such innate peptides can be used to permeabilize the outer membrane of their source bacteria is intriguing, both from a fundamental and a drug discovery perspective. It is also interesting to examine if this feature is conserved among bacterial species.

To test if this feature occurs in Gram-positive bacteria, the membrane-anchoring peptides from the proteins FtsA and MinD derived from the Gram-positive model organism *Bacillus subtilis*, were tested (Strahl, H., personal communication). These peptides showed no membrane activity up to 50 μM. This could be simply due to inactivity of the peptides or the fact that the tested peptides were not C-terminally amidated, a standard modification to increase peptide stability. Another observation was made when the SepF protein, which carries a similar membrane-binding motif, was plasmid-encoded and expressed in *E. coli* (Wenzel, M., personal communication). SepF is a protein unique to Gram-positive bacteria that fulfills a similar function as FtsA in the bacterial cell division[25]. The expression of SepF was unsuccessful at first due to its high toxicity for *E. coli*. However, when the N-terminus was shielded, which is where the membrane-binding domain is located, the expression worked without problem, suggesting that it is this domain that renders SepF expression toxic for *E. coli*.

To investigate further how common the feature of amphipathic α-helices is in the membrane-binding domains of bacterial proteins, a bioinformatic analysis was performed (Bianco, 2020). The *E. coli* peripheral membrane proteome [26] was examined for interesting proteins and a long list of potential proteins was established (Bianco, 2020). This was expanded to other proteins from other bacteria and the candidate proteins were analyzed using Amphipaseek and Heliquest. Amphipaseek is a program that estimates the amphiphilicity of a protein sequence [27]. This gives insight into the likeliness of an amphiphilic α-helix formation of a peptide sequence. Heliquest was then used to estimate the alignment of the amino acids into a potential α-helical configuration of the peptide sequence derived from Amphipaseek [28]. This program also calculates the physicochemical properties of the peptide, such as mean hydrophobicity and mean hydrophobic moment. An example of Amphipaseek and Heliquest analysis is shown in Appendix A Figure A.1, A.2. The resulting list of peptides sequences was used to select peptides for experimental study of membrane permeabilization.

1.7 Aim

The aim of this project was to investigate the use of peptides from the membrane-binding domains of intrinsic bacterial proteins to permeabilize the outer membrane of Gram-negative bacteria. For this purpose, assays had to be established and optimized to enable assessment of the peptides outer and inner membrane permeabilization against *Mycobacterium tuberculosis.*

Chapter 2: Literature review

2.1 Antibiotic administration and Antibiotic resistance

From soil to skin, food and hence faeces, large populations of antibiotic resistant bacteria have been found prevailing the varied ecosystems across the globe. Bacterial populations may possess antibiotic resistance even against the antibiotics that they do not even produce. Oral exposure of such antibiotic resistance bacteria serve as the main channel via which resistant strains are disseminated to humans in their GI tract. [9]

On administration of antibiotics, due to the amplification of pre-existing antibiotic resistant bacterial population and emergence of endogenous resistant strains, the overall resistant bacterial population increases in the gut. [9] This is simply due to the fact that resistant bacteria transfer resistant genes across the bacterial population residing. Through sequencing experiments, it was revealed that generally, β-lactamases and a large number of homologous genes have been found to be transferred most commonly. Resistant genes often form clusters as they are transferred together. [10] Moreover, resistant bacteria have also been found to alter or utilize their existing metabolic machinery to source the resistant determinants. An example of this phenomenon could be the transfer of efflux pumps and immunity genes from the resistant bacteria to other bacteria. Once transferred, efflux pumps and immunity genes become an exclusive source of resistance determinants. [9]

Once emerged, because of the niche fitness, the antibiotic resistant bacteria persist in the gut of the host. Hence, it can be rightly said that dissemination, amplification, emergence and persistence of antibiotic resistant bacteria in the gut of the host, together, contributes to the prevalence of antibiotic resistance in humans. [9]

2.2 Impact of Antibiotic Administration routes on Antibiotic Resistance

As oral exposure serve as the main gateway for antibiotic resistance bacteria to be disseminated to humans, GI tract/gut is primarily considered for determining the impact of antibiotic administration routes on antibiotic resistance. There are two modes via which antibiotics are administered i.e. oral mode of administration and administration via intravenous (IV) mode. But, the rise in antibiotic resistance in the gut directly depends on the type of antibiotic being administered as different antibiotics have different drug excretion routes. For example, in case of ampicillin, ampicillin is excreted mainly via renal routes. So, when ampicillin is administered via IV mode, there is less chance that pre-existing antibiotic resistant bacterial population in the gut will get access to the antibiotic. However, in case of, for example tetracycline, it is excreted both via renal pathways (glomerular filtration) and GI tract (direct elimination and biliary elimination). So, even if tetracycline is injected intravenously, the pre-existing antibiotic resistant bacterial population will somehow get access to the antibiotic. Nevertheless, the oral administration of any antibiotic whatsoever will result in the increase of antibiotic resistance in the gut of the host as pre-existing antibiotic resistant bacterial population in the gut will get the direct access to the antibiotic. So, conclusively it can be said that, the rise in antibiotic resistance in the gut is especially prominent when antibiotics are introduced orally. Moreover, on comparison of ampicillin and tetracycline, the difference in antibiotic resistance between oral and IV administration routes is much more significantly seen for ampicillin than for tetracycline. [9]

On the other hand, when considering infections at locations in the body other than GI tract such as in the bones and spine, intravenous mode of antibiotic administration is preferred "as intravenous antibiotics penetrate tissues quicker and at higher concentrations than oral antibiotics" [11]. However, for majority of cases, oral route of administration is preferred over the intravenous route due to varied advantages such as lower drug cost, absence of cannula-related infections and no need for a health professional or equipment to administer antibiotics. Even if in cases where IV application is needed, switch over to oral antibiotics is recommended as benefits of IV is limited to first few days of treatment only. Furthermore, for cases such as those of infections in the bones and joints where prolonged IV application is recommended, research is going on for determining the possibility of switching over to oral antibiotics too. Despite knowing that switch over to oral antibiotics is necessary, patients in hospitals are often given antibiotics via IV mode. Prolonged use of intravenous antibiotics can also, therefore, result in the increase of antibiotic resistance. [12]

2.3 Some most problematic antibiotic resistant bacteria

Many dangerous gram positive and gram negative bacteria are emerging as potential threats to humans as they are developing alarming antibiotic resistances to some of the most crucial antibiotics and classes of antibiotics. To name some are methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *Staphylococcus aureus* (VRSA), vancomycin resistant *Enterococci* (VRE), penicillin resistant *Streptococcus pneumoniae*, multi-drug resistant (MDR) *Mycobacterium tuberculosis*, multi-drug resistant (MDR) *Clostridium difficile*, multi-drug resistant (MDR) *Pseudomonas aeruginosa* and multi-drug resistant (MDR) *Acinetobacter*. [10]

2.4 Antibiotic use and acceleration of the evolution of bacterial resistance

Antibiotic use has accelerated the evolution of bacterial resistance in many ways [10]:

a) Over prescription of antibiotics by doctors.

b) Over use of antibiotics by general public due to lack of knowledge. People take antibiotics for infections that may not be even caused by bacteria. According to a European survey carried out in 2009, majority of the people were found to have taken antibiotics for influenza, a viral infection, unaware of the fact that antibiotics do not kill viruses.

c) Overly long antibiotic treatment regimens.

d) Over use of antibiotics in animal feed stocks. An example of this can be the over use of glycopeptide antibiotic Avoparcin as a growth inducer in food animals which led to the emergence of Vancomycin Resistant Enterococci (VRE) in Europe.

e) Over use of antibiotics on fruits. Antibiotics such as Oxytetracycline and aminoglycoside antibiotic Streptomycin are being sprayed on fruits like apples and pears in huge amounts.

f) Waste run-off containing antibiotics or antibiotic resistant bacteria from large agro-business plants may serve as a potential means of facilitating the dissemination of resistance elements in varied ecosystems.

Some important strategies employed to curb the overuse of antibiotics and thus accelerating rise in antibiotic resistances are [10][12]:

a) Antibiotic stewardship programs
b) Reductions in antibiotic usage

c) Cycling usage between antibiotic classes

d) Use of combination therapies

2.3 Peptide selection

Peptides were selected based on their physicochemical properties *i. e.* mean hydrophobicity <H>, mean hydrophobic moment <μH> and net charge (z) (Appendix A, Table A.1). The discriminatory factor D was also a strong basis of selecting peptides, where a D≥1.34 estimated an amphipathic peptide in the folded state and, therefore, outer membrane activity. D was calculated using the hydrophobicity and hydrophobic moment of the peptides through the formula D = 0.944 <μH> + 0.33(z). The selected peptides were synthesized as C-terminally amidated derivates to increase their protease stability[13] and are shown in Table 2.1

Name	Sequences	Organisms	Length (amino acids)	Net charge Z	Hydrophobicity <H>	Hydrophobic moment <μH>	Discriminatory factor D
SepF11	MSTLHKVKAYF	*M. tuberculosis*	11	2	0.507	0.397	1.035
SepF14	MSTLHKVKAYFGMA	*M. tuberculosis*	14	2	0.509	0.248	0.894
FtsA11	WFKKLTGWLRK	*M. tuberculosis*	11	4	0.543	0.825	2.099
FtsA13	WFKKLTGWLRKEF	*M. tuberculosis*	13	3	0.548	0.699	1.650
EspI11	QRGWRHWVHAL	*M. tuberculosis*	11	2	0.523	0.675	1.297
Rv1318c helix 1	VRVVRAALRRV	*M. tuberculosis*	11	4	0.287	0.582	1.869
Rv1320c helix 2	LQRGFNRMVEGLR	*M. tuberculosis*	13	2	0.242	0.724	1.343

MinD11	KKGFFSKLFGG	*M. tuberculosis*	11	3	0.369	0.358	1.328
MurG9a	KGVKALLTA	*M. tuberculosis*	9	2	0.391	0.475	1.108
MurG9b	RIAKAVKQA	*M. tuberculosis*	9	3	0.082	0.594	1.551
FtsY13	MSFFKKLKEKITK	*M. tuberculosis*	13	4	0.226	0.600	1.886
FtsY11	FFKKLKEKITK	*M. tuberculosis*	11	4	0.159	0.706	1.986
GlpD9[1]	YTTYRVMAK	*M. tuberculosis*	9	2	0.356	0.319	0.961
PspA12[1]	TIFGRIAQLTKA	*M. tuberculosis*	12	2	0.501	0.491	1.124
PlsB13[2]	MLNGIQKFFAVSW	*M. tuberculosis,* hypothetical	13	1	0.788	0.544	0.844
PlsB9[2]	MLNGIQKFF	*M. tuberculosis,* hypothetical	9	1	0.722	0.821	1.105
PlsB11	RVLGWTWNRLY	*M. tuberculosis*	11	2	0.702	0.446	1.081
PlsB14	MSLWRKIYYNVLNL	*M. tuberculosis*	14	2	0.734	0.529	1.159

1. Not predicted by amphipaseek to be form an amphipathic helix, however it shows similarities to amphipathic helixes in Heliquest
2. From an hypothetical protein of Mtb

Table 2.1 List of peptides

2.4 Nomenclature of the peptides is based on classical denotations of peptides from their derived protein, with the addition of origin organism for easier distinction. As an example, ECFtsY11 is derived from the protein FtsY and the peptide is 11 AAs long, which lead to the peptide name FtsY11. It is from the organism E. coli therefore an EC is added to the peptide name, in this case ECFtsY11. ECFtsA10, ECMreB9, ECFtsA13 and ECAc-WMreB9 have been studied before [22]. They exhibit an outer membrane activity and ECFtsA13 and ECAc-WMreB9 also show activity against the inner membrane. These peptides were included as a proof of concept for the newly established assays. The sequence and length information of the peptides derived from E. coli proteins were selected from Coralie Bianco's List of peptides (Bianco, 2020).

2.5 Mycobacterium cell envelope:

Mycobacterium belongs to phylum *Actinobacteria* of Gram-positive bacteria which Is a very diverse group of bacteria and most of the species have evolved to have an symbiotic relationship with host which includes parasitic and commensalism for example Mycobactria is pathogenic to humans same goes for Nocardia but other genus that come under phylum Actinobacteria for example Bifidobacterium involves bacterial species that lives in gut and also have been proven to be beneficial for health so they are basically part of our system that's not all some bacterial species that come under Actinobacteria are also involved in production of different sort of bioproducts like Streptomyces sp. are involved in antibiotic compound production[29].

Mycobacterium which is one the most studied organism from phylum *Actinobacteria* is known for having a unique and taxonomic diverse cell envelope as they have an additional arbinogalactan layer which connects the outer membrane or "mycomembrane" to the peptidoglycan layer in addition of a plasma membrane and a cell wall, presence of this cell envelope which is not usual in nature makes mycobacterium unique because of this cell envelope mycobacterium can survive several external stress and physiological conditions and this cell envelope is also responsible for virulence, one of the most popular species of *Mycobacterium* which is an etiological agent for tuberculosis is *Mycobacterium tuberculosis*, tuberculosis is one of the leading cause of death in all over the world and according to WHO controlling tuberculosis is one of the major priority worldwide [30]. The so called "mycomembrane" present in the cell envelope of organisms of order *Corynrbacteriales* which

also includes M. tuberculosis has shown resemblance towards the outer membrane of Gram-negative bacteria[31][32].

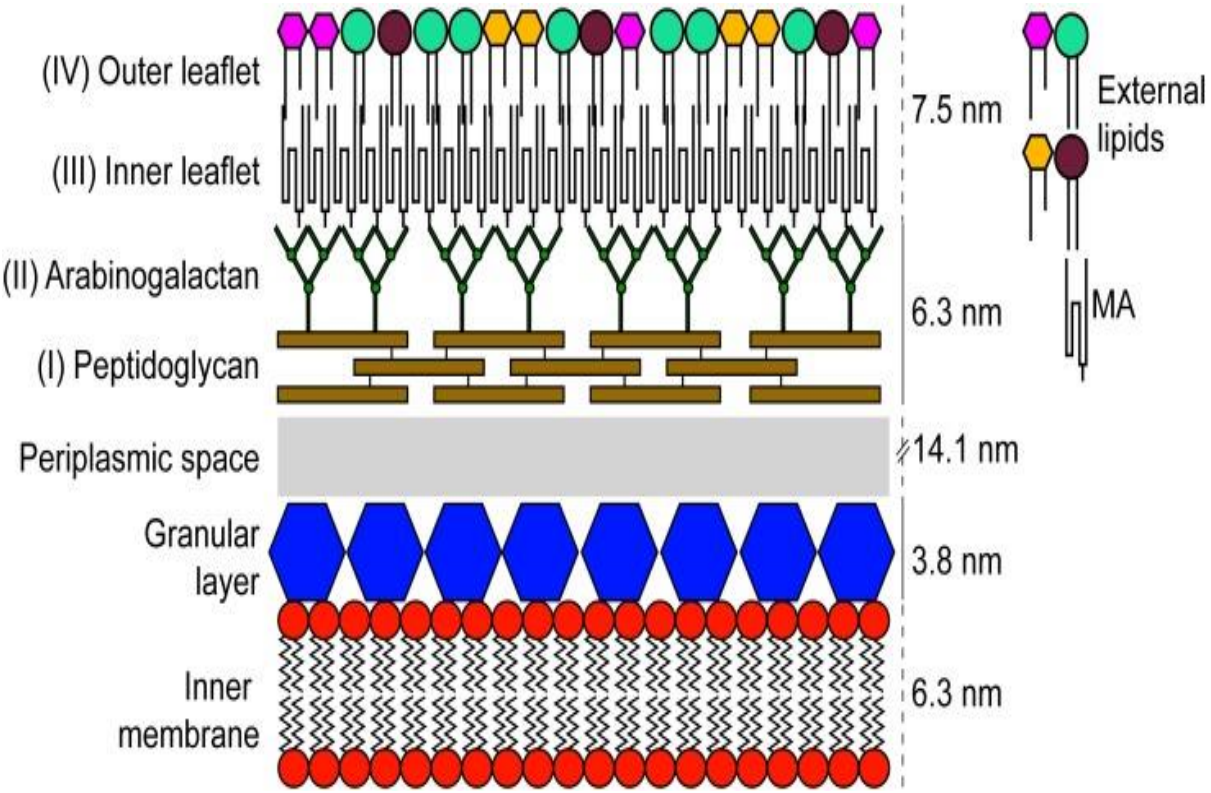

Fig 2.1 A schematic diagram showing a typical cell envelope of organisms belong to Corynebacteriales [33].

Layer 1 : peptidoglycan layer is the first layer, present in all Actinobacteria it generally serves the function of providing rigidity and also help in maintenance of osmototic stability.

Layer 2 : second layer shown in the diagram is Arabiogalactan, it is a heteropolysaccharide which is highly branched made up of monomers galactose and arabinose forming a furanoid

Layer 3 : Mycolic acid makes up for the third layer where the external mycomembrane is composed of mycolic acids which is a fatty acid with long chains, this is very particular to the *Corynebacterials* [4]. This mycolic acid layer is responsible for the preventing the entry of antibiotics and several other hydrophilic molecules into the cell by acting like a barrier but this layer is also the target for major anti-tuberculosis drugs, it can be said that mycolic acid layer plays an important role in differentiation of organism on taxonomic level within the order *Corynebacterials* as not every member of this order have same sort of functional groups or

number of carbons present in the this layer of mycolic acid hence it becomes easier to classify them among genera and species[34].

Layer 4 : lipids present externally over the cell envelope are placed into this fourth layer, this is basically the outer part of mycomebrane which is very heterogenous in nature as it consist of lipids, proteins and other molecules like lipoglycans[35].

2.6 Membrane permeabilization

To allow passage of smaller molecules (≤600 Da), the outer membrane is perforated by porins. These are pore-forming proteins that constitute channels between the periplasmic space and the extracellular space. There are many different forms of porins, but they usually fold into β-barrel structures [36]. Porins exhibit varying passage mechanisms, that can be non-specific or specific towards size and charge of solutes [37]. In order to determine the selectivity of the peptides, the outer and inner membrane permeability need to be assayed. In this thesis, the assays used for this purpose were the NPN assay, DiSC3(5) assay, MinD delocalization assay, and ML-35p assay.

2.7 Outer membrane permeabilization

The outer membrane-permeabilizing activity of the peptides can be assayed using *N*-phenyl-1-naphtylamine (NPN) (Loh *et al*., 1984). NPN is a hydrophobic molecule with a weak fluorescent signal in aqueous solutions, but a stronger signal in hydrophobic environments, like lipid membranes[38] [39]. NPN itself cannot traverse the outer membrane, which ensures that intact cells are not stained. An increase in the fluorescent signal is therefore an indication of permeabilizing activity of the peptides. This can be studied with excitation at ~350 nm and emission at ~420 nm.

2.8 Membrane depolarization

The inner membrane permeabilization can be assayed with DiSC3(5), which is a voltage-sensitive molecule with fluorescent properties[4]. Due to the membrane potential, DiSC3(5) amasses at the membrane surface. This displays as a strong fluorescing cell, when observed under a fluorescence microscope. When the membrane is permeabilized and consequently depolarized, DiSC3(5) is released into the medium causing a decrease in fluorescence intensity over the cell.

Chapter 3: Materials and methods

3.1 Materials

Material	Manufacturer
Ampicillin	Fisher bioreagents
Costar Microtiter plate (96-well, polystyrene, black, clear round bottom, non-treated)	Corning
Dimethyl sulfoxide (DMSO)	Duchefa Biochemie
3,3'-Dipropylthiadicarbocyanine Iodide (DiSC3(5))	Anaspec Inc.
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Fisher BioReagents
Isopropyl β-D1-thiogalactopyranoside (IPTG)	Sigma-Aldrich
Microtiter plate (96-well, polystyrene, clear flat bottom, non-treated, sterile)	Falcon
Mueller Hinton broth (MHB)	Sigma-Aldrich
Nitrocefin	Sigma-Aldrich
2-Nitrophenyl β-D-galactopyranoside (ONPG)	Sigma-Aldrich
N-phenyl-1-naphtylamine (NPN)	Sigma-Aldrich
Peptides	Pepscan, Lelystad, Netherlands
Phosphate buffered saline (PBS)	Sigma-Aldrich
Polymyxin B (PolB)	Duchefa Biochemie
Polymyxin B nonapeptide hydrochloride (PMBN)	Sigma-Aldrich
Sodium chloride (NaCl)	Promega
Tryptone *Biochemica*	ITW Reagents
Yeast extract	Duchefa Biochemie

Table 3.1. Chemicals and material

3.1.1 Growth media recipes

The growth media used were medium and Mueller-Hinton broth (MHB) and Middlebrook 7H9 broth base. The recipes for the media are shown in Table 3.2, 3.3, respectively.

Material	**Amount**
Mueller Hinton Broth	21 g
MilliQ-water	1 L

Table 3.2. Mueller Hinton Broth recipe

Material	**Amount** (g/L)
Ammonium sulfate	0.50
Biotin	0.0005
Disodium phosphate,	2.50
Monopotassium phosphate,	1.00
Sodium citrate	0.10
Magnesium sulfate	0.05
Calcium chloride	0.0005
Zinc sulfate	0.001
Copper sulfate,	0.001
Ferric ammonium citrate	0.04
L-Glutamic acid,	0.50
Pyridoxine	0.001

Table 3.3 Middlebrook 7H9 broth base ingredients

Recipe: 2.3 g of Middlebrook 7h9 Broth Base was suspended into 450ml of distilled water followed by addition of 0.5 g of TWEEN 80 then it was sterilised by autoclaving for 15 min, after cooling below 45 degrees Middlebrook ADC Growth Supplement was added. This media is generally stored in fridge at 4 degrees.

3.1.2 Assay buffers

The assay buffers used were 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and phosphate buffered saline (PBS) and the recipes are shown in Table 3.4 and 3.5, respectively.

Material	Amount
HEPES	2 g
MQ	1 L

Table 3.4 HEPES buffer recipe

The buffers were adjusted with NaOH or HCl to pH 7.4. Buffer was filter sterilized through 0.2 μm filters.

3.2 Equipment

The plate readers used were FLUOstar OPTIMA and CLARIOstarPLUS, BMG LABTECH.

3.3 Minimal inhibitory concentrations

Minimal inhibitory concentration (MIC) of the peptides was determined using the broth microdilution standard method (Clinical and Laboratory Standards Institute, 2020).
E. coli MC4100 was inoculated in 2 mL LB medium or 2 mL MHB and incubated overnight at 37 °C, with constant shaking (200 rpm). The cells were diluted in media and grown to a desired OD600=1. A 96-microwell plate was prepared with sterile media by adding 100 μL of the media to the desired wells, including a growth curve control and sterile control illustrated in Figure 3.1. 100 μL of additional media was added to the sterile control in column 1 (Fig. 3.1) and one set of sample wells (column 11, Fig. 3.1). To the latter, PolB, PMBN and the peptides were added at a concentration of 512 μg/mL. 100 μL from each well in column 11 (Fig 1.), were transferred to adjacent wells creating 2-fold serial dilutions of the compounds from 512 μg/mL to 1 μg/mL (Fig. 3.1). The last 100 μL was added to a sterile control for the peptides in Row G (Fig. 3.1). The bacterial culture was adjusted to a turbidity of 1*106 CFU/mL in 14 mL media. 100 μL of the diluted bacteria was added to all wells in column 12 to column 2 (Fig. 3.1), which resulted in the final concentrations of the peptides ranging from 256-0.5 μg/mL. The bacteria in the wells had a final CFU count of 5*105 CFU/mL. The microplate was measured for 16.5 hours in a plate reader at 37 °C. The experiment was done

in triplicates for all peptides in both LB medium and MHB for *E.coli*. for Mycobacterium the plate is incubated for 2 days that is 48 hours in an incubator and then an end point reading was taken, the reason behind this was that Mycobacterium grows super slow hence it generally takes 2-3 days for it to grow and then only we can take the reading.

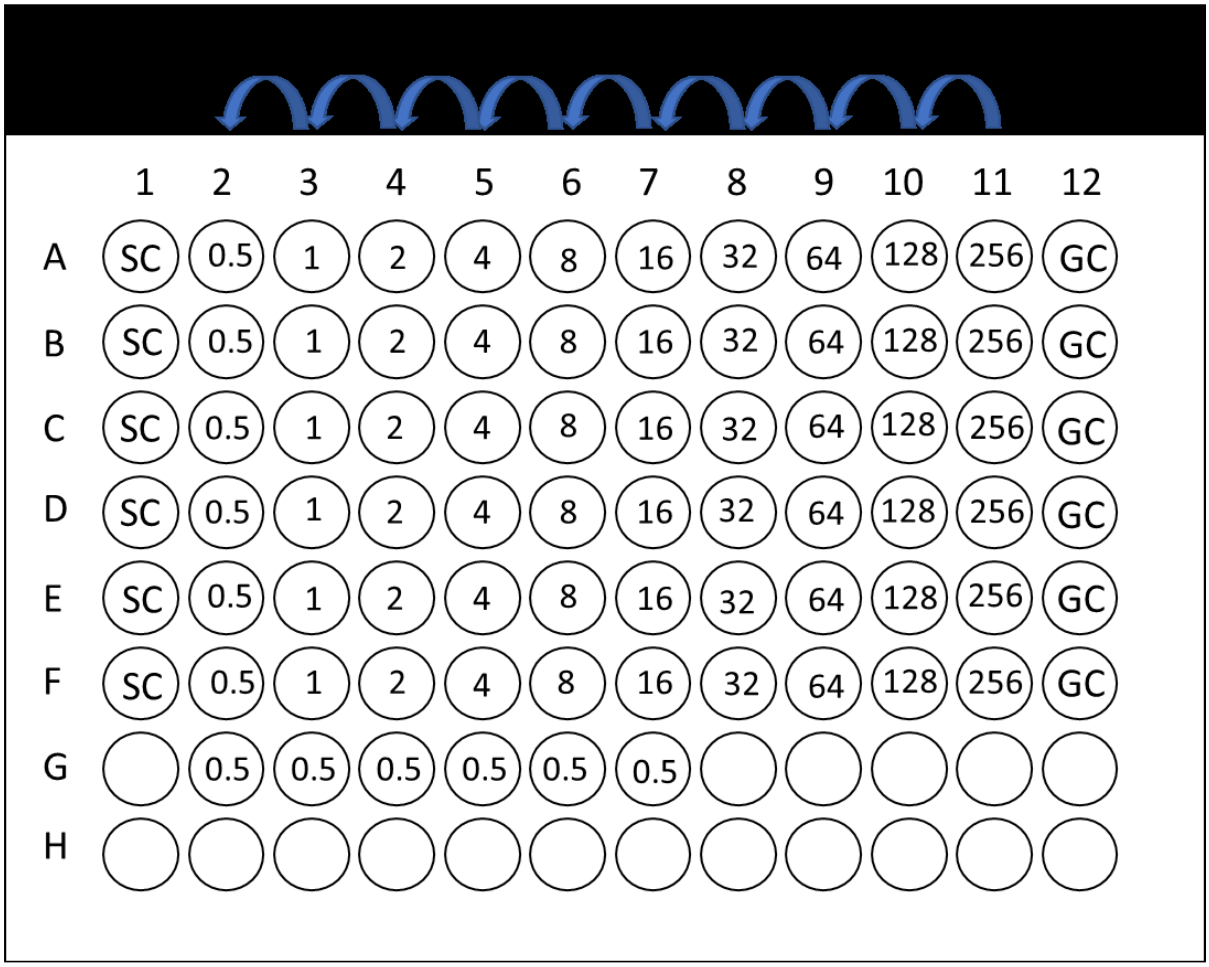

Figure 3.1 Schematic plate used for MIC (Falcon, 96-well sterile plate).

SC stands for sterile control, which is 200 µL media. GC is growth curve of 5*105 CFU/mL bacterial cells in media.

Numbers 256-0.5 refers to concentration of compounds in µg/mL, with one compound tested per row.

Row G was used as sterile control for compounds at 0.5 µg/mL in media.

3.4 Outer membrane permeabilization assay

The peptide-induced permeabilization of the outer membrane of *E. coli* MC4100, was determined using the probe 1-*N*-phenylnaphtylamine (NPN). NPN has a low fluorescent signal in aqueous solution but exhibits an increased fluorescence intensity in hydrophobic environments such as the lipids in the inner leaflet of the outer membrane[40].

3.4.1 Assay development

NPN was prepared for the assay at a concentration of 150 or 300 μM NPN in HEPES from a stock solution of 5 mM NPN in ethanol according to Table 3.6. PolB at 0.1, 0.5 and 1 mg/mL was prepared as shown in Table 3.6. The PolB concentrations correspond to final concentrations of 1, 5 and 10 μg/mL.

Solute	**HEPES**	**MQ**	**5 mM NPN**	**10 mg/mL PolB**	**1 mg/mL PolB**	**Total volume**
300 μM NPN	178.6 μL	-	11.4 μL	-	-	190 μL
150 μM NPN	184.6 μL	-	5.4 μL	-	-	190 μL
1 mg/mL PolB1	-	9 μL	-	1 μL	-	10 μL
0.5 mg/mL PolB1	-	5 μL	-	-	5 μL	10 μL
0.1 mg/mL PolB1	-	9	-	-	1 μL	10 μL

1 PolB concentrations corresponds to final concentrations of 10, 5 and 1 μg/mL.

Table 3.5 Prepared solutions for outer membrane permeabilization assay

E. coli MC4100 was grown overnight in 2 mL LB medium at 37°C with constant shaking of 200rpm. The cells were diluted and grown to mid-log phase (OD600=0.4-0.6). The cells were harvested and washed in 5 mM HEPES buffer pH 7.4 at 14000 rpm for 1.5 min at 37 °C. The cells were resuspended in HEPES buffer (5 mM, pH 7.4) to an OD600=0.5.

A microtiter plate (96-well, Costar) was placed on a heating block at 37 °C and addition to the plate was done according to Table 3.7.

Sample	HEPES	Cells	NPN	PolB (1mg/ml)	PolB1	MQ	Total Volume
Blank	148.5 µL	-	-	-	-	1.5 µL	150 µL
Cells	10 µL	138.5 µL	-	-	-	1.5 µL	150 µL
Negative control	-	138.5 µL	10 µL	-	-	1.5 µL	150 µL
Interaction control2	138.5 µL	-	10 µL	1.5µL	-	-	150 µL
Interaction control2	138.5 µL	-	10 µL	-	1.5 µL	-	150 µL
Positive control	-	138.5 µL	10 µL	1.5 µL	-	-	150 µL
Sample wells	-	138.5 µL	10 µL	-	1.5 µL	-	150 µL

1PolB was either 0.5mg/mL or 0.1 mg/mL.
2 Interaction control refers to control for interaction between NPN and PolB.

Table 3.6. Plate additions for outer membrane permeabilization assay development

HEPES buffer (5 mM, pH 7.4) and cells were added to the plate (Table 3.7) and the baseline was determined for ~5 cycles at 30 s per cycle in a plate reader. The plate reader had a settling time of 0.2 s with 30 flashes per well and ran for a total of 30 cycles at a constant temperature of 37 °C. The excitation filter was set to 355 nm and the emission was measured at 410 nm, with slit width of 10 nm for both filters. 10 µL of 150 µM NPN was added to the wells (Table 3.7) and the reading was continued for ~5 cycles to allow the fluorescent signal of NPN to stabilize. PolB at 1, 0.5 or 0.1 mg/mL was added to the plate and the reading was continued for 10 cycles. All wells were done in technical replicates of three.

3.4.2 NPN assay

NPN was prepared at a concentration of 150 µM in 5 mM HEPES buffer from a stock solution of 5 mM NPN in 100% ethanol (Table 3.8). PolB and peptides were prepared at solutions of 1 mg/mL from stock solutions of 10 mg/mL (Table 3.8).

Solute	HEPES	5mM NPN	0.5mm NPN	Peptide stock	MQ	Total volume
0.5 mM NPN	900 µL	-	100 µL	-	-	1 mL
150 µM NPN	2100 µL	-	-	900 µL	-	3 mL
PolB	-	9 µL	-	-	1 µL	10 µL
Peptides	-	9 µL	-	-	1 µL	10 µL

1, Stock solutions for peptides and PolB had a concentration of 10mg/mL

Table 3.7 Prepared solutions for outer membrane permeabilization assay

Overnight culture of *E. coli* MC4100 grown in 2 mL MHB (37 °C, 200 rpm) was diluted in 3 mL MHB and grown to mid log phase (OD600=0.4-0.6). The cells were washed (14000 rpm, 1.5 min, 37 °C) and resuspend in 5 mM HEPES buffer pH 7.4 to OD600=0.5.

A 96-well plate was placed on a thermo block at 37 °C. 5 mM HEPES buffer pH 7.4 and the cells were pipetted into the corresponding wells according to Table 3.9.

Sample	HEPES	Cells	NPN (150 µM)	PolB (1mg/ml)	Peptide (1mg/mL)1	MQ	Total Volume
Blank	148.5 µL	-	-	-	-	1.5 µL	150 µL
Negative control	-	138.5 µL	10 µL	-	-	1.5 µL	150 µL
Interaction control2	138.5 µL	-	10 µL	1.5µL	-	-	150 µL
Interaction control2	138.5 µL	-	10 µL	-	1.5 µL	-	150 µL
Positive control	-	138.5 µL	10 µL	1.5 µL	-	-	150 µL
Sample wells	-	138.5 µL	10 µL	-	1.5 µL	-	150 µL

1 Three peptides were assayed per plate.
2 Interaction control refers to control for interaction between NPN and PolB as well as for each peptide.

Table 3.8 Plate addition of compounds for NPN assay

3.4.3 Examination of the NPN plate

The plate was examined using a plate reader with bottom optics and ran for 30 cycles at 30 s per cycle. The settling time was 0.1 s with 50 flashes per well. The excitation wavelength was set to 350 nm and emission wavelength was measured at 405 nm, with 10 nm slit width for both. The temperature of the plate reader was kept at 37 °C.

For *E.coli* The baseline of HEPES and cells was measured without the presence of NPN nor peptides for 5 cycles. 10 µl of 150 µM NPN was added to the wells and the plate was measured for 5 cycles to ensure the fluorescent signal of NPN had stabilized before addition of the peptides.

For *Mycobacterium smegmatis* the experimental procedure was a modified, all the materials and basic procedure was entirely similar as for E.coli but since Mycobacterium is considered as BSL2 organism plate was prepped by adding all the ingredients at once under the hood by maintaining a window of 2- 3 mins where addition of cells was the last thing to be added into the plate and then the plate is incubated for 15 mins at 37 degrees in a incubator then reading was taken in the plate reader. The measurement for this NPN assay was a end point reading taken at same parameters as of *E.coli.*

NPN uptake was calculated for each peptide using Equation 1.

$$\% \text{ NPN} = [\text{Fobs} - \text{F0F100} - \text{F0}] \times 100 \quad (1)$$

Where Fobs is the last observed fluorescence of the given peptide, F0 is the last fluorescence of cells with NPN and F100 is the last fluorescence intensity of cells treated with 10 µg/mL PolB. All wells were done in replicates of three and each experiment was repeated three times.

Chapter 4: Results

4.1 Minimal inhibitory concentrations

The antibacterial activity of the peptides was tested against the Gram-negative bacterium *E. coli* and the Gram-positive bacterium *B. subtilis.* Peptides that are selective for the outer membrane will likely show no or low activity (high MICs) against *E. coli*. Peptides were originally tested against *E. coli* grown in LB medium. These experiments displayed no MICs for most peptides. Therefore, the peptides were tested against *E. coli* grown in MHB, which contains lower concentrations of NaCl. The MIC values of the peptides against the bacteria are shown in Table 4.1

Peptides	MIC concentrations
SepF11	>256
SepF14	>256
FtsA11	256
FtsA13	256
EspI11	64
Rv1318c helix 1	>256
Rv1320c helix 2	>256
MinD11	>256
MurG9a	>256
MurG9b	>256
FtsY13	>256
FtsY11	>256
GlpD9[1]	>256
PspA12[1]	>256
PlsB13[2]	>256
PlsB9[2]	>256
PlsB11	>256
PlsB14	>256

Table 4.1. Minimum inhibitory concentration of peptides against *E.coli*

All the peptides have shown higher MICs in E.coli grown in MHB medium, the possible reason could be that either they have higher MICs as they are derived from Gram positive Bacteria. Only peptides FtsA 11 have shown a MIC at 256 which is a high concentration then peptide FtsA13 have shown a MIC at 256 same as of FtsA11 and the last MIC was seen in EspI 11 at 64 which is a low concentration in comparison of other peptides that have shown a MIC but definitely a higher concentration. The reason could be that as these peptides have derived from a gram positive bacteria that is mycobacterium and tested over a gram negative bacteria.

PolB was tested together with every plate of peptides as a positive control and it have shown a MIC ranging from 0.5 to 0.00025.

Pol N which is similar to polB but it have nonapeptide hence the name polB nonapeptide was also tested in the same MIC plate as another control to test the integrity of cells and the MIC was obtained at 128 which is quite high but since we already have polB as a control we just considered the result obtained with polB.

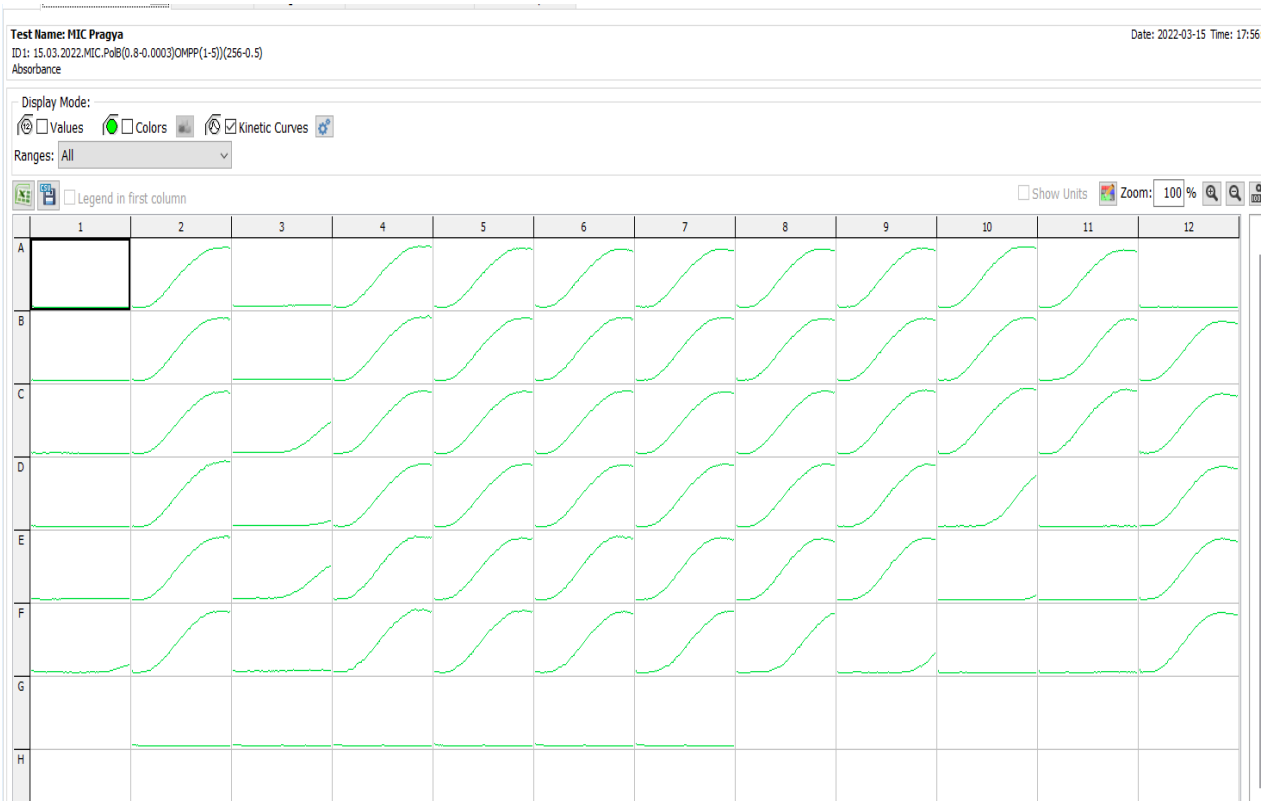

Fig 4.1: an example of MIC result after 16 hours of incubation in the plate reader, this kinectic curves were used to identify the MICs in *E.coli.*

MIC result in mycobacterium was a little bit different as, the incubation of mycobacterium was done in an incubator and then just the end pint reading was taken to know the MIC, so there kinetic curves were not considered to identify the MIC but rather the readings were used.

	01	02	03	04	05	06	07	08	09	10	11	12
A	0.384	0.817	0.986	1.073	1.007	0.985	1.33	1.004	1.082	1.382	1.077	0.964
B	0.425	0.902	0.938	0.838	1.111	1.17	1.041	1.01	0.699	1.044	0.648	1.057
C	0.47	1.431	0.687	0.322	0.477	0.234	0.795	0.253	0.59	0.234	0.371	0.261
D	0.56	0.305	0.522	0.274	0.333	0.61	0.322	0.501	0.321	0.299	0.597	0.582
E	0.321	0.594	0.306	0.309	0.493	0.29	0.61	0.229	0.63	0.367	0.346	0.598
F	0.35	0.313	0.299	0.577	0.36	0.586	0.355	0.643	0.359	0.605	0.384	0.546
G	0.402	0.33	0.385	0.299	0.69	0.341	0.805	0.32	0.401	0.298	0.456	0.315
H		0.599	0.46	0.568	0.585	0.702	0.568	0.357				

Table 4.2 MIC Endpoint result for Mycobacteria using peptides SepF11, SepF14, FtsA11, FtsA13, Espl11, Rv1318c and Rv1320c and PolB.

All the result for Mycobacteria smegmatis was taken like this where the endpoint reading was compared, if the reading of any well is more than the sterile control its is considered as MIC and polB ie polymyxin B is used as a positive control which is same as of E.coli.
The result for Mycobacteria is very different from the result for E.coli all the results are summarized in table 4.3

Peptides	MIC concentration
SepF11	256
SepF14	64
FtsA11	128
FtsA13	64
EspI11	8
Rv1318c helix 1	256
Rv1320c helix 2	>256
MinD11	>256
MurG9a	>256
MurG9b	>256
FtsY13	>256
FtsY11	>256
GlpD9[1]	>256
PspA12[1]	>256
PlsB13[2]	>256
PlsB9[2]	>256
PlsB11	>256
PlsB14	>256

Table 4.3 MIC result in *Mycobacterium smegmatis*

MIC for Mycobacteria is quite different from what we observed in *E.coli*. MIC is observed in SepF11 at 256, SepF14 at 64, FtsA11 at 128, FtsA13 at 64, EspI11 at 8 and Rv1318 at 256 which is very strange and completely distinct from the result in Gram negative bacteria.

If we compare the result of both Gram positive bacteria and gram negative bacteria fro the same peptides its easier to draw the differentiation between the behaviour of these peptides for both type of organisms, the comparison can be seen in table 4.4

Peptides	**MIC conc. *E.coli***	**MIC conc. In *M.smegmatis***
SepF11	>256	256
SepF14	>256	64
FtsA11	256	128
FtsA13	256	64
EspI11	64	8
Rv1318c helix 1	>256	256
Rv1320c helix 2	>256	>256
MinD11	>256	>256
MurG9a	>256	>256
MurG9b	>256	>256
FtsY13	>256	>256
FtsY11	>256	>256
GlpD9[1]	>256	>256
PspA12[1]	>256	>256
PlsB13[2]	>256	>256
PlsB9[2]	>256	>256
PlsB11	>256	>256
PlsB14	>256	>256

Table 4.4 Minimum inhibitory concentration of peptides against Gram-positive and Gram-negative bacteria.

4.2 Outer membrane permeabilization assay

The peptides were then tested for outer membrane-permeabilizing properties against the outer membrane of *E. coli* MC4100 using the fluorescent probe NPN. NPN exhibits an increased fluorescence in hydrophobic environments such as the lipids residing in the membrane of bacteria. NPN cannot permeate the outer membrane of Gram-negative bacteria. Therefore, an increased fluorescence is an indication of outer membrane permeabilization.

4.2.1 Assay development

To assess the ability of the peptides to permeabilize the outer membrane of *E. coli,* the NPN assay had to be established. This was executed with testing of NPN concentrations and varying concentrations of PolB, which was to be used as positive control. PMBN was also tested to examine how a selective outer membrane-permeabilizing peptide could behave in this assay.

As described before in section 3.3.1, *E. coli* MC4100 culture growing in log phase, was washed and resuspended in HEPES buffer (5 mM, pH 7.4). The cells were transferred to a microtiter plate and a baseline fluorescence of cells and blanks was measured in a plate reader. 10 µM was added to cells and buffer alone and after the fluorescent signal of NPN stabilized, the cells were treated with 1, 5 or 10 µg/mL PolB (Fig 4.1a, b). PMBN at 5 and 10 µg/mL was also tested (Fig 4.1b). 20 µM NPN was also tested (not shown), but this increased the fluorescence in untreated cells and consequently decreased the gap in fluorescence intensity between PolB and untreated cells. Therefore, 10 µM NPN was established as the optimum concentration. Interaction controls between NPN and PolB or PMBN were also tested at the highest concentration of peptide (Appendix B, Fig B.1). There was no significant interaction observed between the peptides and NPN. However, it is still an important control to include since interactions between fluorophore and peptide can give false positive results.

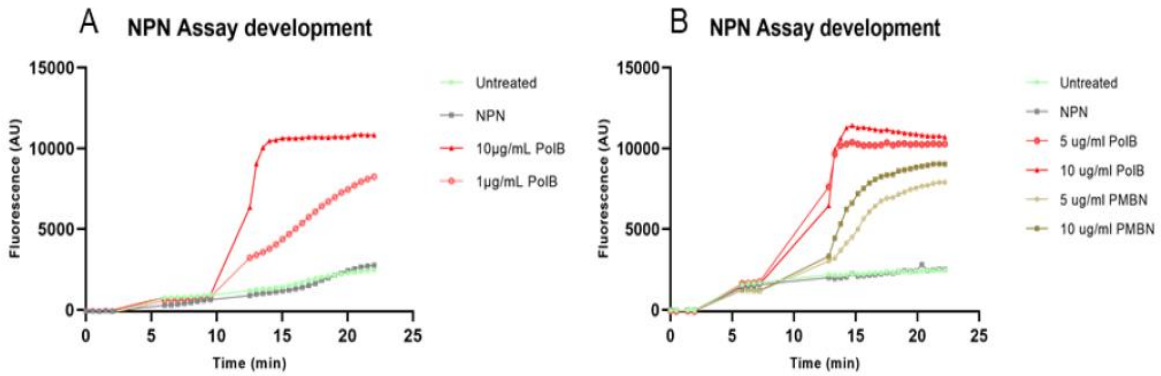

Figure 4.2. Outer membrane permeabilization assay development. E. coli MC4100 cells treated with Polymyxin B (PolB) or Polymyxin B nonapeptide (PMBN) in the presence of 10 µM NPN. NPN exhibits a low fluorescence intensity in aqueous solution and shows an increased fluorescence in lipid environments. (A) Cells treated with 10 µg/mL PolB show a rapid NPN uptake with maximum value reached ~1 min after first measurement. 1 µg/mL PolB shows a slow uptake of NPN by the cells. (B) Cells treated with PolB at 10 and 5 µg/mL show

rapid NPN uptake with maximum reached within minutes. PMBN at 10 and 5 μg/mL shows an uptake of NPN caused by outer membrane permeabilization of the cells with increase in fluorescence intensity throughout the measurement time. Untreated cells for both A and B are E. coli MC4100 at OD600=0.5 in 5mM HEPES buffer pH 7.4 containing 10 μM NPN. NPN refers to 10 μM NPN alone in buffer. Interaction between NPN and PolB as well as PMBN was tested. However, no significant fluorescent increase was observed.

Cells treated with 10 μg/mL PolB exhibited a rapid increase in fluorescent intensity, reaching a maximum value ~1 min after measurement start (Figure 4.1A, B). This is caused by almost instant permeabilization of the outer membrane of the cells. 1 μg/mL PolB (Fig. 4.1A) and PMBN (5 and 10 μg/mL, Fig 4.1B) had a continuous increasing fluorescence intensity throughout the measurement. 10 μg/mL PolB was selected as the optimum concentration to use as positive control when testing the peptides.

4.2.2 NPN Assay

As described before in section 3.3.2 and 4.2.1, *E. coli* MC4100 was tested in HEPES buffer (5 mM, pH 7.4). 10μM NPN was added to cells and buffer alone and after the fluorescent signal of NPN stabilized, the cells were treated with 10 μg/mL peptide. 10 μg/mL PolB was used as positive control and negative control was cells with NPN. Peptide-promoted NPN uptake was measured for 10 min and percentage uptake was calculated (Fig 4.1). The NPN uptake caused by 10 μg/mL PolB was used as 100% NPN uptake.

	1	2	3	4	5	6	7	8	9	10	11	12
A	2557	2913	2419	2419	2544	2637	2597	2447	2353	2416	2316	2189
B	2496	2411	2376	2359	2419	2438	2450	2375	2291	2237	2235	2353
C	2397	2462	2453	31267	22763	23427	25315	30395	32046	30210	32173	31809
D	31102	29150	34462	34185	29597	31025	37780	31576	34771	31329	37481	34192
E	31129	32213	31095	32225	31949	31738	31644	27281	27565	22101	27603	28439
F	27528	27702	27853	24378	44773	29746	39098	31719	32896			

Table 4.5 NPN assay end point reading result for *Mycobacterium smegmatis* (cycle 1)

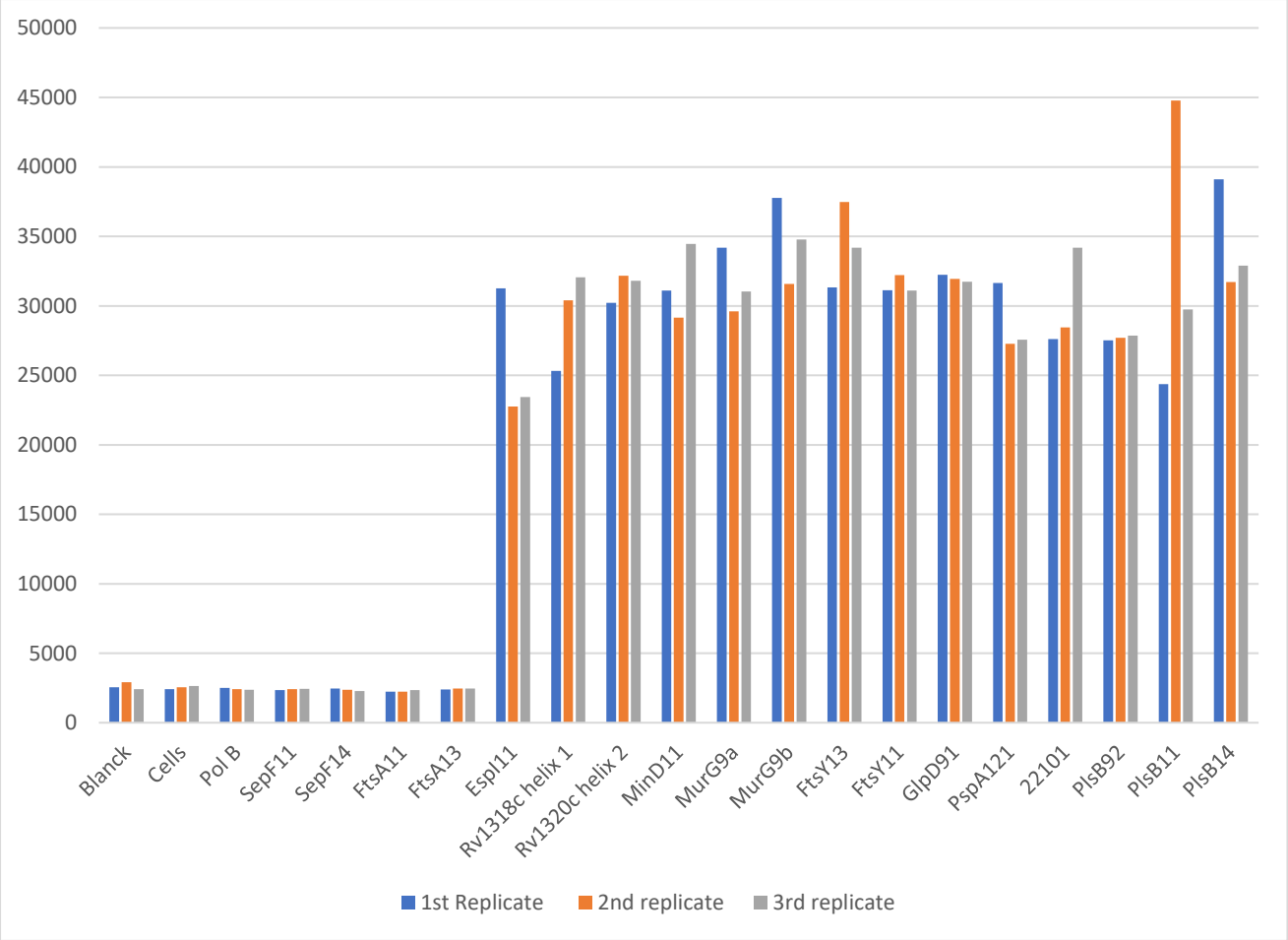

Figure 4.3 Graphical representation of result of NPN assay in *Mycobacterium smegmatis* (cycle 1) The cells were treated with 10 µg/mL peptide in the presence of NPN. The increase in fluorescence as NPN enters the lipid environments of the cell envelope was measured. Cells treated with 10 µg/mL Polymyxin B was taken as 100% NPN uptake. Bars show biological replicates, where each replicate is the average of three technical replicate wells.

	1	2	3	4	5	6	7	8	9	10	11	12
A	5142	6179	4437	2609	3459	4653	28006	26833	28098	10706	11484	12621
B	25786	22985	20175	23296	24309	19771	27440	28072	20964	21035	22761	25724
C	25015	25512	26054	27789	28029	28821	27107	32877	32202	26769	28375	30729
D	25370	27614	27622	27509	28501	27416	29834	29052	29608	28782	28045	22768
E	25410	24788	26495	27345	27801	29784	28105	26940	26625	28202	27009	28429
F	24866	27357	29300	22250	29488	26304	27328	27873	27857			

Table 4.6 NPN assay end point reading result for *Mycobacterium smegmatis* (cycle 2)

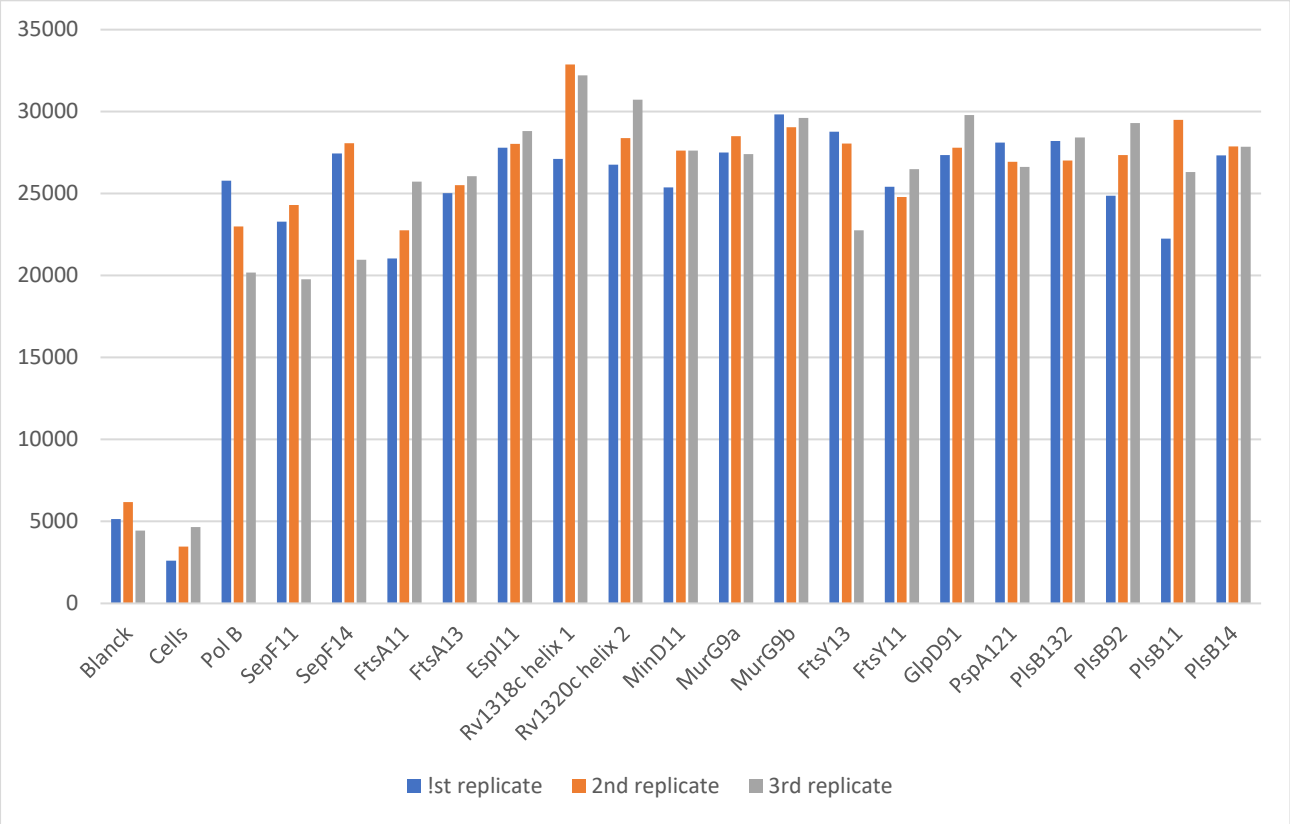

Figure 4.4 Graphical representation of result of NPN assay in *Mycobacterium smegmatis* (cycle 2 The cells were treated with 10 µg/mL peptide in the presence of NPN. The increase in fluorescence as NPN enters the lipid environments of the cell envelope was measured. Cells treated with 10 µg/mL Polymyxin B was taken as 100% NPN uptake. Bars show biological replicates, where each replicate is the average of three technical replicate wells.

	1	2	3	4	5	6	7	8	9	10	11	12
A	11194	12069	12021	5298	5519	5280	20897	23262	24693	13267	12833	11622
B	27984	27518	30286	27720	28513	26167	27192	25658	27874	25397	25552	28095
C	31189	26739	27400	24754	26559	26479	26551	49882	23895	23752	26356	26228
D	27176	27440	24996	27172	24197	26731	24359	22290	25290	23980	27565	32697
E	23859	20899	20492	22221	17578	23367	20331	21244	21867	21502	23214	19645
F	24483	23092	22987	20872	21271	18353	26510	22147	3035			

Table 4.7 6 NPN assay end point reading result for *Mycobacterium smegmatis* (cycle 3)

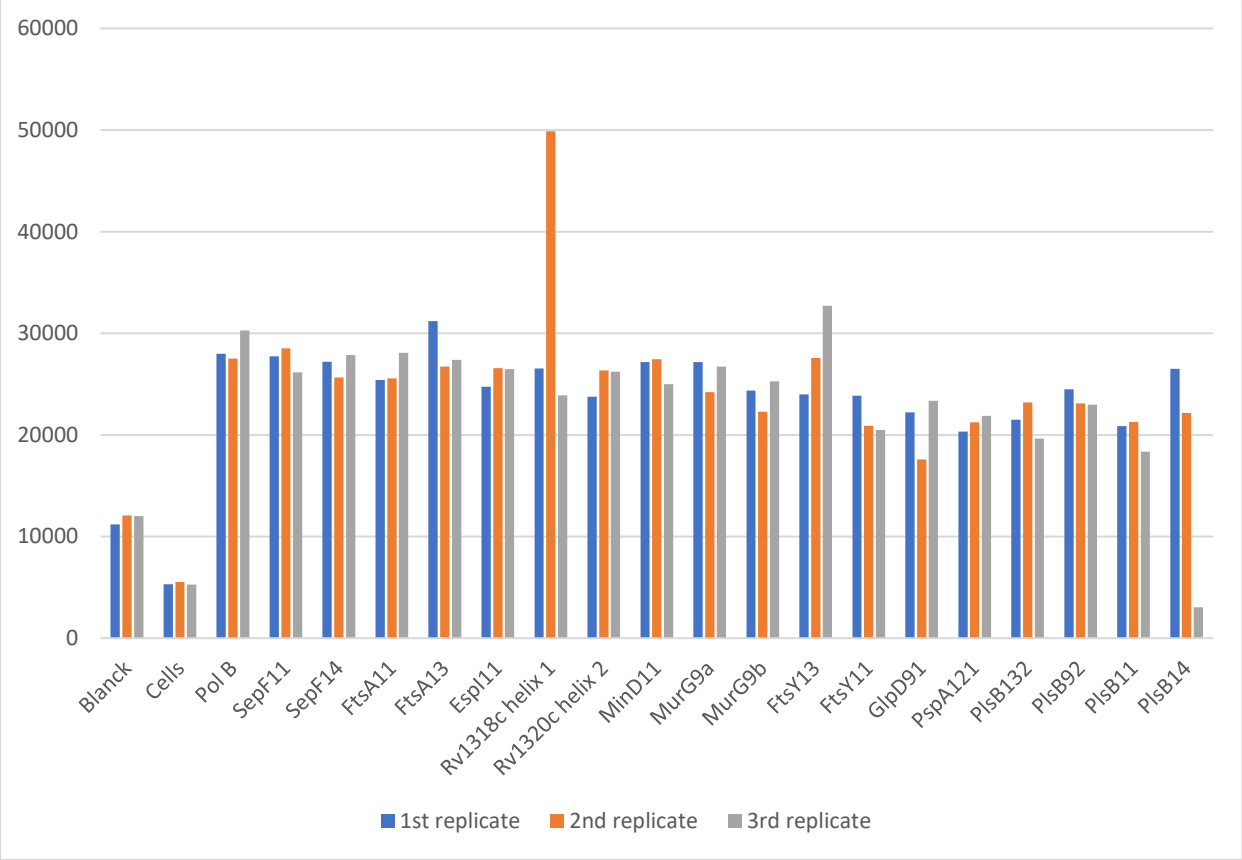

Figure 4.5 Graphical representation of result of NPN assay in *Mycobacterium smegmatis* (cycle 3)

The cells were treated with 10 µg/mL peptide in the presence of NPN. The increase in fluorescence as NPN enters the lipid environments of the cell envelope was measured. Cells treated with 10 µg/mL Polymyxin B was taken as 100% NPN uptake. Bars show biological replicates, where each replicate is the average of three technical replicate wells.

Chapter 5: Discussion

It has been proven that intrinsic bacterial peptides can be used against their native bacteria [24]. At the same time, the two peptides FtsA10 and MreB9 were found to only cause permeabilization of the outer membrane. These peptides showed no activity towards the inner membrane and had low antibacterial activity [22]. This sparked the idea to continue the search for selective outer membrane acting peptides in the bacterial proteome. The search was focused on membrane binding domains of innate protein sequences and the occurrence of amphipathic α-helices in these regions.

Many intrinsic proteins were bioinformatically investigated (Bianco, 2020), and potential amphipathic peptides were selected. In this thesis, 14 peptides were tested for antibacterial activity, outer and inner membrane activity. Four of these peptides had been studied before, three were peptides from *B. Subtilis* and seven were from *E. coli*.

5.1 Minimal inhibitory concentration

Minimal inhibitory concentrations were used to test the antibacterial activity of the peptides against *E. coli* and *Mycobacterium smegmatis.* Most peptides showed no visible MIC at the highest concentration tested (256 μg/mL) against *E. coli* grown in MHB medium. This could be explained with the difference in NaCl concentration between the two media. Salt has been found to affect peptides antibacterial activity before[41][33]. This could be due to Cl- interacting with the cationic peptides, which hinders the peptide from interacting with the membrane. It could also be that the Na+ competes with the peptides for interaction with the negatively charge lipopolysaccharides in the outer leaflet of the outer membrane of *E. coli*. Another explanation is that salt provides osmotic membrane stabilization [42].

Many of the peptides displayed low activity against *E. coli* in MHB. This was not surprising as peptides that exhibit selective outer membrane activity does not necessarily show antibacterial activity. This has been shown by Vaara and Vaara to be the case for PMBN [16][39]. M. Vaara *et al*. also showed this for another polymyxin B derivates called NAB7061. They saw that NAB7061 had low activity (high MICs) against the Gram-negative bacteria tested, but could sensitize the bacteria to five antibiotics that are usually inactive against them.

5.2 NPN assay

The fact that most peptides showed a lower MIC against *mycobacterium smegmatis* implies that none of the peptides are selective for the outer membrane. SepF11 showed low activity in *E.coli* but showed activity in *M. smegmatis* although the outer membrane of the mycobacteria is tough to cross but still there was inhibition in growth of *Mycobacteria* so it might be possible that SepF11 is able to cross the outer membrane barrier. FtsA11 which is selective to the outer membrane [16], displayed high MICs (low activity) against both *E. coli* and *M.smegmatis*. This gives further indication that none of the peptides are outer membrane selective. However, some peptides did display selective outer membrane activity against *E. coli,* which was confirmed by several assays (Table 4.6). All the peptides expect SepF11, SepF14 showed an effect only in the NPN Assay. This is not in line with these peptides exhibiting antibacterial activity against *E.coli.* This indicates that these peptides could have a different secondary target in *M.smegmatis* compared to *E. coli.* Whether this target is the cytoplasmic membrane or another target, such as teichoic acids [18], remains to be assessed in future studies. Interesting to note is also that SepF11 was the only peptide showing increased MICs (lower activity) against *M. smegmatis*, compared to the other Gram-positive derived peptides, SepF14, FtsA11, FtsA13 and EspI11 which showed increased activity against *M. smegmatis*. This is in line with the previously observed toxcicity of the SepF membrane-binding domain in *E. coli* (Wenzel, M., personal communication) and is an interesting aspect to be pursued further in the future.

Except SepF11 and SepF14 all the peptides have shown a increased in NPN signal in Mycobacterium smegmatis which shows that they are able to permeabilize the outer membrane of this Gram-positive bacteria which is very interesting and can be used in further studies and can be continued with further experiments that will prove if they actually can used as antimycobacterial peptides utilized for actual clinical applications.

Chapter 6: Conclusion

Research regarding AMPs tends to focus on antibacterial activity and inner membrane activity. However, AMPs with selective outer membrane-permeabilizing properties could be used in combination with many existing antibiotics to treat multi-drug resistant bacterial infections. This project was focused on examining peptides from membrane-binding domains of innate proteins. The peptides were thought to form amphipathic α-helices, which confers activity towards outer and inner membrane. I have demonstrated that the bacterial proteome is host to AMPs that can be used as antibacterial and membrane-perturbing compounds against their native organisms as well as other bacteria.

The aim of this project was divided into two parts; the first part was a method development, where four different assays were assessed, and three were established and fully optimized. The second part was to investigate membrane binding domains of proteins from the bacterial proteome, to find peptides with outer membrane permeabilization properties against Gram-negative bacteria, by utilizing the established assays.

The data shows that the bacterial proteome is host to many peptides that is active against both inner and outer membrane of Gram-negative bacteria. Six out of 14 peptides were shown to be selective for the outer membrane in *E. coli.* Moving on there should be further tests with different peptide concentrations and variations of peptide sequences for the assays established. Furthermore, the effect of varying salt concentrations against the peptides should be tested. In addition, hemolytic and cytotoxicity assays should be performed to assess the safety of the peptides and their potential as future antibiotic potentiators. It would also be interesting to see the effects of the peptides against pathogenic gram-negatives and mycobacteria. Lastly, the research should be expanded to innate peptides from other organisms. The fact that this thesis found innate bacterial peptides with a variety of effects towards bacteria, including membrane activity and different antibacterial activity shows that the bacterial proteome can be used as a source for a vast variety of bioactive molecules and can therefore be studied in a broader perspective as well, aside from finding outer membrane selective peptides.

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Appendix A

```
QQGVKHHLAAGIVLTGGAAQIEGLAACAQRVFHTQVRIGAPLNITGLTDYAQEPYYSTAVGLLHYGKESHLNGEAEVEKR

ccccccccceeeeeccccchhhhhhhhhhhhcccccccccccccccccccccccceeeeeeeccccccccccccc
222112212122111112222222343343344443322122122233333221112222222222221111233223


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         AAAAAAAAAAA
ccccccchhhhhhhhhhccc
33344444444555444344
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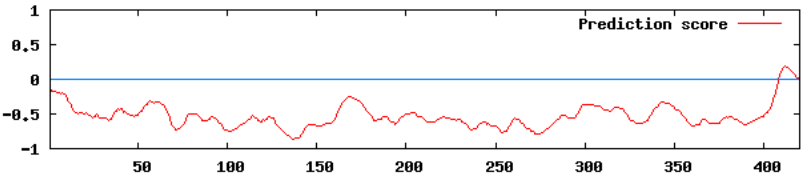

Figure A.1. Amphipaseek result of the C-terminus of the protein FtsA10. The sequence of FtsA was taken from *E. coli* K-12. The red A:s show a predicted amphipathic sequence. The prediction score for each amino acid in the sequence indicates amphipathicity.

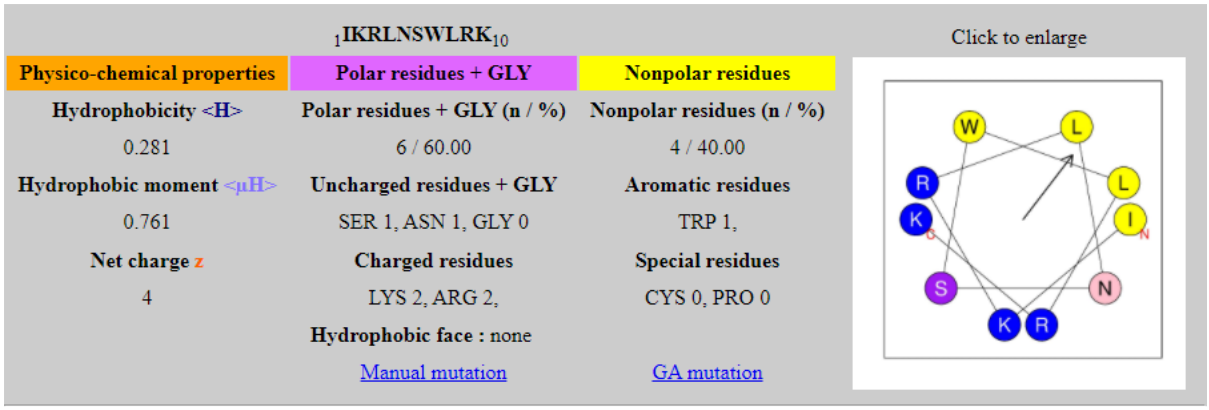

Figure A.2. Heliquest results of the peptide predicted to be amphipathic by Amphipaseek. The predicted α-helix of the FtsA peptide shown on the right. Heliquest predicts physicochemical properties of the peptide.

Appendix B

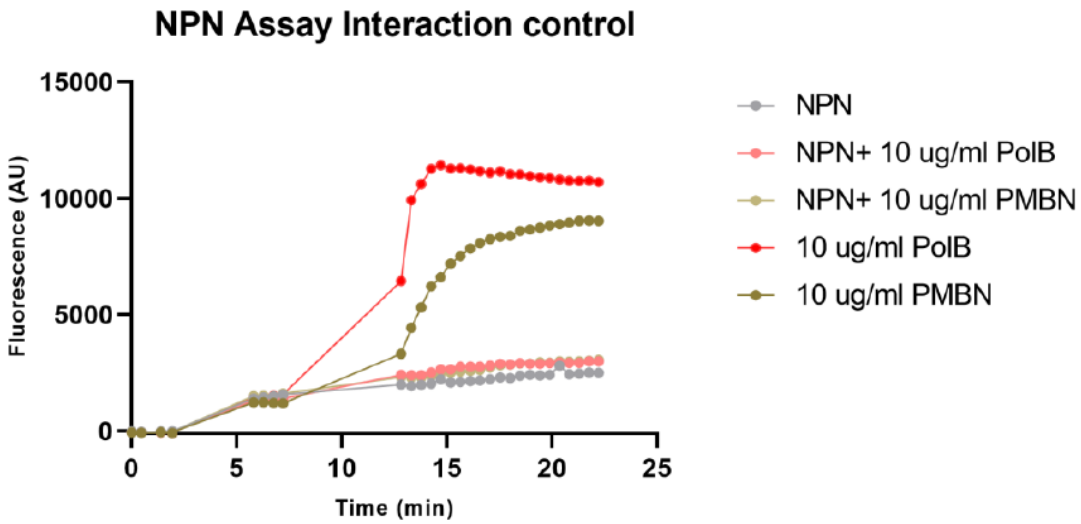

Figure B.1 Interaction control between NPN and PolB or PMBN.

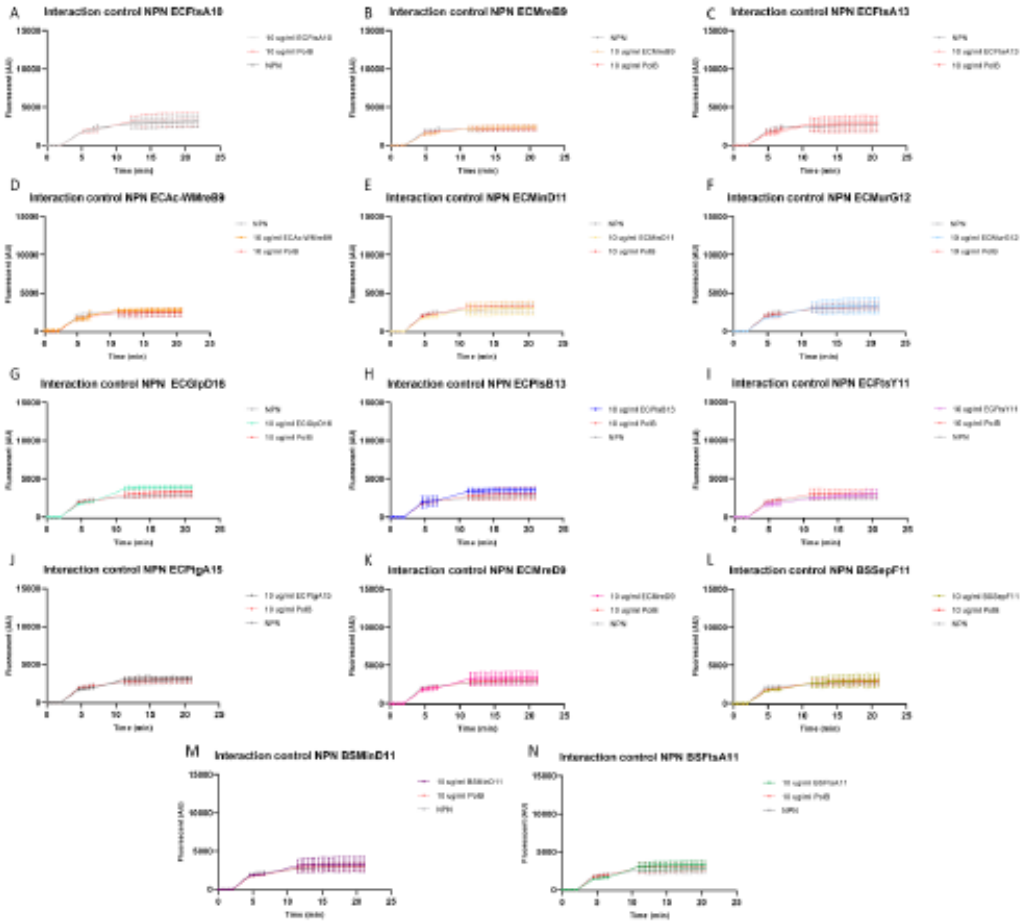

Figure B.4 Interaction control between NPN and innate peptides in HEPES buffer.

CANDIDATE'S DECLARATION

I, Pragya Kamal, Roll No. 2K20/MSCBIO/21 M.Sc. Biotechnology student declares that the project report titled "Breaking the intrinsic antibiotic resistance -Outer membrane permeabilizing peptides from innate bacterial proteomes" which has been submitted to the Department of Biotechnology, Delhi Technological University, New Delhi, is original and has not taken from any of the sources without proper citation. This work has not previously formed the basis for the award of any Degree, Diploma Associateship, Fellowship or other similar title or recognition.

PLACE: Delhi

Date: 05 May, 22

Pkamal

PRAGYA KAMAL
2K20/MSCBIO/21

CERTIFICATE

I hereby certify that the dissertation project titled **"Breaking the intrinsic antibiotic resistance -Outer membrane permeabilizing peptides from innate bacterial proteomes"** which is submitted by Pragya Kamal, 2K20/MSCBIO/21, Department of Biotechnology, Delhi Technological University, Delhi in partial fulfilment of requirement for the award of the degree of Master of Science, is a record of work carried out by the student under my supervision. To the best of my knowledge this work has not been submitted in part of any Degree or Diploma to this University or elsewhere.

Date: 02-05-2022

Place: Sweden

Yours Sincerely,

Michaela Wenzel,
Assistant Professor, Chemical Biology Division

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CERTIFICATE

I hereby certify that the dissertation project titled **"Breaking the intrinsic antibiotic resistance -Outer membrane permeabilizing peptides from innate bacterial proteomes"** which is submitted by Pragya Kamal, 2K20/MSCBIO/21, Department of Biotechnology, Delhi Technological University, Delhi in partial fulfilment of requirement for the award of the degree of Master of Science, is a record of work carried out by the student under the supervision of Dr. Michieala Wenzel, Chalmers University of Technology, Sweden. I trust the statement of Dr. Wenzel as she states that this work has not been submitted in part of any Degree or Diploma to this University or elsewhere.

Place: Delhi

Date: 05/05/2022

Prof. Jai Gopal Sharma

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Pkamal

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