Antimicrobial Peptides from Membrane-Interacting Stretches of Bacterial Proteins

A thesis submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

BY

KARABI SAIKIA

Department of Biosciences and Bioengineering

Indian Institute of Technology Guwahati

Guwahati - 781039, India

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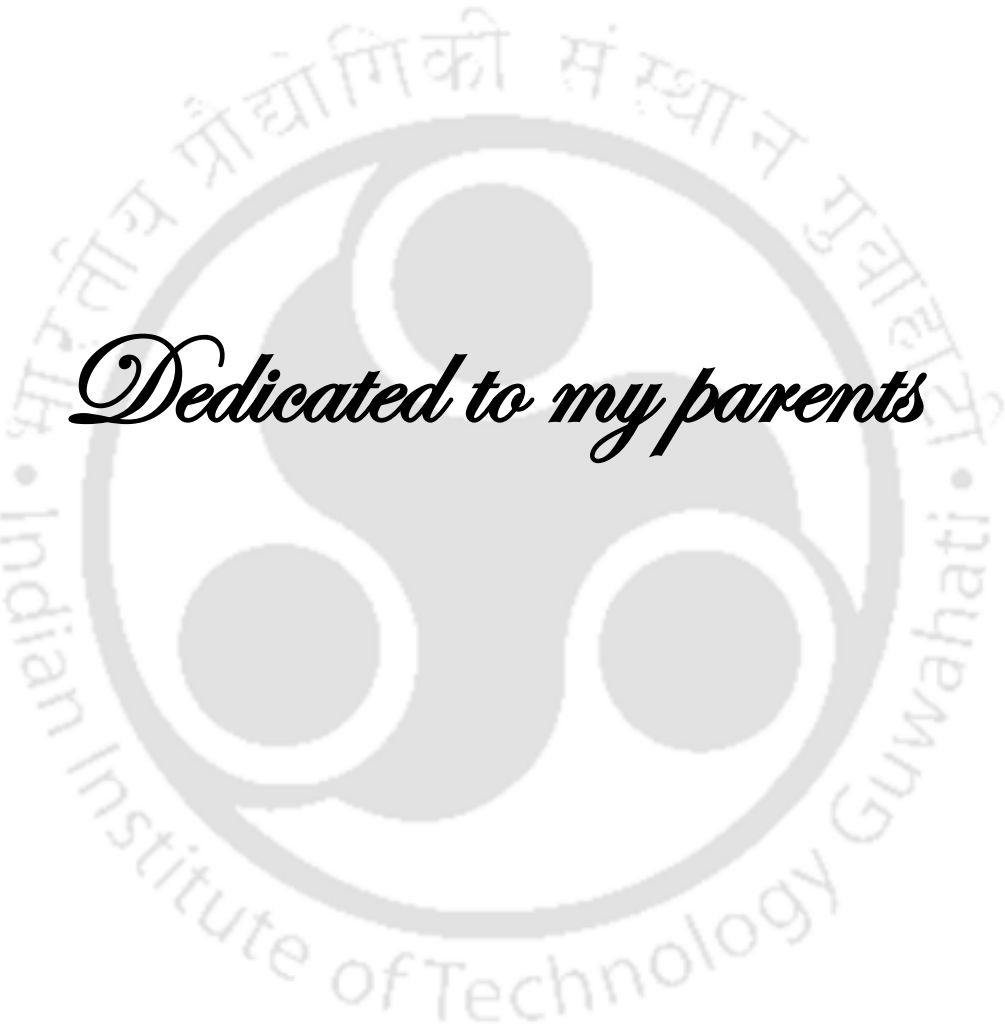

Dedicated to my parents

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INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI
DEPARTMENT OF BIOSCIENCES AND BIOENGINEERING

DECLARATION

The research work presented in this thesis is an original work carried out by me in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India, for the degree of the Doctor of Philosophy. Research work was executed under the guidance of my supervisor Dr. Nitin Chaudhary, and no work has been submitted, in part or whole, for any other degree to any other University/Institute. As per the standards of reporting research findings, due acknowledgements have been made wherever the work described is based on the findings of other researchers.

Karabi Saikia

Date:

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CERTIFICATE

This is to certify that the work incorporated in the thesis titled "**Antimicrobial Peptides from Membrane-Interacting Stretches of Bacterial Proteins**" submitted by Ms Karabi Saikia for the award of the degree of Doctor of Philosophy is an authentic record of the research work carried out under my guidance in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India. The work is original and has not been submitted in part or full for any other degree of any other University/Institute.

Dr. Nitin Chaudhary

(Supervisor)

Date:

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Abbreviations

AMP	Antimicrobial peptide
CHL	Cholesterol
DIPEA	N,N-diisopropylethylamine
$DiSC_3(5)$	3,3′-dipropylthiadicarbocyanine iodide
DMF	Dimethylformamide
EDTA	Ethylenediaminetetraacetic acid
Fmoc	Fluorenylmethyloxycarbonyl chloride
FESEM	Field emission scanning electron microscope
HBTU	N,N,N',N'-tetramethyl-O-(1H-benzotriazol-1-yl)uranium hexafluorophosphate
HCCA	α-Cyano-4-hydroxycinnamic acid
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HOBt	1-hydroxybenzotriazole hydrate
HPLC	High performance liquid chromatography
M	Molar
MALDI-TOF	Matrix-assisted laser desorption ionization-time of flight
MDR	Multidrug resistant
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *S. aureus*
NPN	N-phenyl-1-naphthylamine
OD	Optical density
POPC	1-palmitoyl-2-oleoyl-glycero-3-phosphocholine
POPE	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine
POPG	1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol)
SUVs	Small unilamellar vesicles
TB	Tuberculosis
TFA	Trifluoroacetic acid
TFE	Trifluoroethanol
XDR	Extensively drug resistant

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Above all, I thank my family for their love, affection, and trust on me. I don't have enough words to express my gratitude towards my parents for giving me the freedom to chase my dreams, my husband Devabrat, who stood by me in all my ups and downs, and my sisters Aditi and Manjeera whose love and support was immeasurable in my journey. My research would not have been possible without their encouragement and blessings.

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Synopsis

Higher organisms have cohabited with microorganisms on this planet throughout their evolution and have devised strategies to control and combat them. Antimicrobial peptides (AMPs) happen to be one of the key components of such defense arsenal and play an important role to ward off pathogens [1]. AMPs occur naturally in almost all organisms as an important element of their innate immune system [2]. AMPs have been exposed to microbes for millions of years, but resistance against them is not prevalent. In fact, mutations in microbes during the course of evolution have led to the diversification of AMPs [1]. The sequence diversity of AMPs is such that classifying them based on their sequences is neither practical nor of much use. Such diversity in AMPs could account for the inability of microbes to develop good resistance against them thereby letting such an ancient weapon flourish throughout evolution. The activity of peptides can be altered by making subtle changes in their amino acid sequence, composition, and conformation, suggesting that AMPs hold promise to be developed into next-generation antibiotics [3].

Cationic antimicrobial peptides (CAMPs) *i.e.* the peptides possessing a net positive charge at neutral pH, constitute the largest group of AMPs [4]. Structurally, these peptides could attain diverse conformations such as α-helices, β-sheets, mixed conformations, loops, and extended structures [5]. An important feature of AMPs is that they fold into amphipathic structures that could interact with bacterial membranes [6]. Amphipathic α-helical peptides constitute the largest structural class among all known CAMPs [7].

In this thesis, I sought to investigate the antimicrobial potential of the peptide stretches that cause tethering of bacterial proteins to membranes. For this purpose, membrane-interacting peptide stretches were identified from two *E. coli* proteins *viz.* MreB and FtsA. In addition to that, antimicrobial properties of a peptide derived from LCI, a 47-residue *B. subtilis* AMP effective against plant pathogens, are

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investigated. The thesis is divided into six chapters and the contents of these chapters are briefly summarized below.

Chapter 1. Introduction and Literature Review

The chapter gives a general overview of antimicrobial peptides. It comprises an introduction to AMPs, their historical perspective, structural diversity, and different mechanisms of their action. The chapter also discusses various immunomodulatory functions of AMPs, the challenges that AMPs face to be developed as antibiotics, and some of the peptides that made into clinical trials or are in use.

Chapter 2. Materials and Methods

Chapter 2 describes the materials and methodologies used for carrying out the thesis work. The chapter includes protocols for solid phase peptide synthesis, peptide purification and characterization, antimicrobial assays, membrane-binding and membrane-permeabilization assays, hemolytic assay, electron microscopic imaging, and various biophysical techniques utilized for the thesis work.

Chapter 3. Antimicrobial peptides from N-terminal membrane-binding region of *E. coli* MreB protein

The chapter describes the short AMPs derived from N-terminal membrane-binding region of *E. coli* MreB protein. The peptides are 9 and 10-residue long, possess a net positive charge, and preferentially bind to negatively charged lipid vesicles. The peptides display broad-spectrum activity, killing Gram-negative bacteria, Gram-positive bacteria, as well as fungus. The peptides cause permeabilization of *E. coli* membranes indicating membrane-permeabilization as one of the mechanisms of killing.

Chapter 4. Antimicrobial peptides from C-terminal amphipathic region of *E. coli* FtsA protein

Incited by the results obtained with MreB-derived peptides, I investigated the antimicrobial potential of 10, 11, an 13-residue peptide-stretches lying in C-terminal amphipathic helix of *E. coli* FtsA protein. The 11 and 13-residue peptides turn out to

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be potent antimicrobials. Like MreB-derived peptides, the peptides display preferential binding to negatively charged lipid vesicles and permeabilize both outer and inner membrane of *E. coli*. Interestingly, the peptides display selectivity towards Gram-negative bacteria.

Chapter 5. Aromatic residue-rich antimicrobial peptide derived from C-terminal region of *B. subtilis* LCI peptide

LCI is a 47-residue AMP produced by *B. subtilis* that inhibits plant pathogens. The peptide folds into a four-stranded antiparallel β-sheet and is believed to exhibit antibacterial activity by interacting with membranes. The peptide lacks disulfide linkages but displays an unusually high thermodynamic stability that is believed to be conferred by aromatic interactions. As 80% aromatic residues lie in the C-terminal 26-residues, I studied the antimicrobial potential of LCI_{22-47}. This chapter includes the data from these studies. The peptide killed *E. coli*, *X. oryzae* pv. *oryzae*, and gentamicin-resistant MRSA at very promising concentrations. The peptide selectively permeabilizes the outer membrane of *E. coli* indicating the formation of transient pores.

Chapter 6. Conclusion, Discussion, and Possibilities

The chapter concludes the thesis by discussing the highlights of results described in chapters 3-5. The results, the future possibilities, and interesting but unexplored questions are discussed. In addition, preliminary data for the anti-mycobacterial activity of most potent MreB and FtsA-derived peptides are included to support the hypothesis and discussion. To our surprise, the peptides killed *M. smegmatis* at concentrations as low as 4 μM even in the presence of salt and divalent cations.

Membrane-binding peptide stretches in microbial proteins, therefore, could be a good source of novel AMPs.

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List of publications

1. Saikia, K.; Sravani, Y. D.; Ramakrishnan, V.; Chaudhary, N., Highly potent antimicrobial peptides from N-terminal membrane-binding region of *E. coli* MreB. *Sci Rep* **2017,** *7*, 42994.

2. Saikia, K.; Chaudhary, N., Interaction of MreB-derived antimicrobial peptides with membranes. *Biochem Biophys Res Commun* **2018,** *498* (1), 58-63.

3. Saikia, K.; Chaudhary, N., Antimicrobial peptides from C-terminal amphipathic region of *E. coli* FtsA. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **2018,** *1860* (12), 2506-2514.

Manuscript under preparation

1. Saikia, K., Belwal, V., Datta, D., Chaudhary, N. (2018). Aromatic-rich C-terminal region of LCI is a potent antimicrobial peptide in itself.

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CHAPTER 1

Introduction and Literature Review

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1.1 Introduction

Evolution and natural selection are the fundamental laws of nature. Living organisms evolve by retaining the beneficial traits and discarding the detrimental ones. Microorganisms that harbor antibiotic-resistance genes would outcompete the drug-susceptible microbes in an antibiotic-containing medium. The period between the 1950s to 1970s is considered the golden age of antibiotics; a large number of antibiotics were discovered during this period and the otherwise fatal bacterial infections could easily be treated [8]. The antibiotic-resistant bacteria, however, flourished under the antibiotic selection pressure. The problem is not restricted to bacteria; other organisms like fungi, viruses, and parasites have also acquired drug-resistance. Drug-resistance is a well-acknowledged medical threat. According to 2018 World Health Organization (WHO) report on antimicrobial resistance, some of the common, potentially harmful organisms are becoming more aggressive by acquiring drug resistance. By the end of 2016, around 4,90,000 people were reported globally with multidrug-resistant tuberculosis (MDR-TB), a form of tuberculosis that is resistant to two most-powerful anti-TB drugs. Gradually, MDR-TB strains evolved into extensively drug-resistant (XDR) ones causing XDR-TB, a form of tuberculosis that is resistant to at least four of the major anti-TB drugs. The discovery of new antibiotics has lagged behind, and antibiotic resistance has become a very serious threat [9, 10]. Discovery of a novel class of drugs therefore is the pressing need to meet this challenge. Non-conventional antimicrobials are drawing interest to be utilized as alternatives to antibiotics [11]. Peptides constitute one such class of molecules and hold promise to be the next generation antibiotics [3]. Antimicrobial peptides, usually abbreviated as AMPs, are indigenous constituents of the innate immune system in a wide variety of organisms like insects, plants, animals, and microorganisms to fend off pathogens [2, 12-15]. Antimicrobial peptides are the most ancient and efficient host defense weapons that exhibit activity against microbes ranging from Gram-positive and Gram-negative bacteria, viruses, fungi, and protozoa [12, 16]. Some peptides are also reported to have anti-cancer and anti-biofilm activity [17-20]. Other than displaying a direct antimicrobial killing, many AMPs function as important effectors and regulators of the innate immune system as well thereby modulating the immune responses [15, 21]. AMPs

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are mainly gene encoded; a majority of bacteria, however, synthesize their AMPs non-ribosomally [22]. One of the most imperative factors for any antimicrobial agent is its selectivity for pathogens; the pathogen should be combated without adversely affecting the host cells. Bacterial cell surfaces are negatively charged due to the presence of anionic phospholipids, lipopolysaccharides, and teichoic acid in their membranes. Mammalian cells, on the other hand, are composed of zwitterionic lipids. It is this difference in the cell surface that AMPs utilize for selectively targeting bacterial cells. A large majority of AMPs are cationic in nature *i.e.* they harbor lysine and arginine residues that impart them positive charge at neutral pH [5]. The net positive charge on AMPs causes them to preferentially bind to the anionic bacterial surfaces. In a membrane-like environment, most AMPs take up a conformation that imparts them amphipathicity; membrane-perturbation by these amphipathic entities results in cell death [2]. More than 2000 AMPs have been isolated from a diverse range of organisms, and a large majority is isolated from the animal kingdom followed by plants (Figure 1.1) [23].

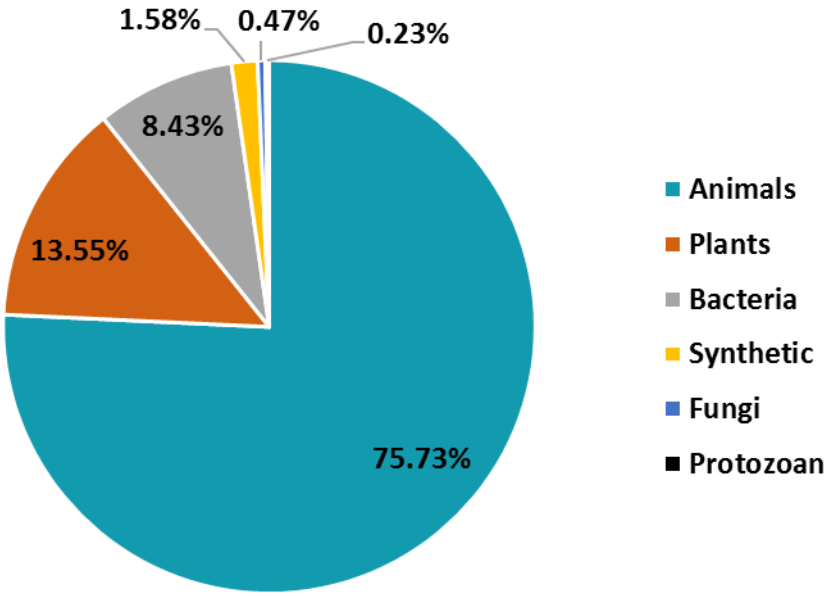

Figure 1.1 Abundance of AMPs in various life forms [23]

1.2 Distinctive features of antimicrobial peptides

Charge: Majority of the antimicrobial peptides discovered till date are cationic in nature; anionic antimicrobial peptides, however, are also known. Positive charge can be imparted

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by the cationic residues lysine and arginine as well as the free amino-terminus. The net charge on AMPs usually ranges from +2 to +9 [5]. The positive charge plays an important role in the initial electrostatic interaction of peptide with membrane. Moreover, transmembrane potential of prokaryotic cells is greater than mammalian cells and facilitates the attraction of cationic peptides towards the membrane [24].

Amphipathicity: Most cationic AMPs exhibit their antimicrobial activity through membrane-permeabilization. Such activity is imparted by the amphipathic nature of these peptides wherein hydrophilic and hydrophobic residues are spatially separated. The peptides may or may not be structured in aqueous solutions; however, they take up amphipathic structures upon membrane interaction [25].

Hydrophobicity: Peptide hydrophobicity is an essential requirement for the activity of cationic AMPs. Hydrophobic residues contribute to the amphipathicity of peptides. Approximately 50% of the residues present in AMPs are hydrophobic in nature [26]. Most cationic AMPs display selectivity towards Gram-positive bacteria. Malanovic and Lohner have recently carried out statistical analysis of the known AMPs to understand the AMP selectivity towards bacteria [16]. The analysis indicates a correlation between the peptide hydrophobicity and bacterial selectivity. Peptides that act against Gram-negative bacteria are less hydrophobic (≤50% hydrophobic residues) [16].

Size: The size of AMPs ranges from as small as dipeptides to over 100 residues. The dipeptide AMP, gageotetrin A has only recently been identified and is actually a lipopeptide [27]. The other lipopeptides of the same class *viz.* gageotetrin B and gageotetrin C are tetrapeptides. Interestingly, all these peptides possess a net negative charge at neutral pH. Two and four-residue anionic lipopeptides like gageotetrin and five-residue peptides like cathepsin G (1-5) [28], EP5-1 [29], and plantaricin ZJ008 [30] are among the smallest AMPs. Most AMPs, however, are much longer; the average length of the 3018 peptides listed in APD3 is 32.74 residues [23].

Conformation: The sequences of AMPs are so diverse that they are seldom classified based on their sequences. The AMPs are essentially classified based on their structures. Alpha-helical AMPs constitute the largest structural class. The other common structural classes are discussed in the next section [26].

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1.3 A historical perspective

Discovery of AMPs dates back to early 20th century when Alexander Fleming, while working in his laboratory in 1922, accidentally discovered a bacteriolytic substance present in human nasal secretion. The substance that came to be known as lysozyme displays activity against non-pathogenic bacteria and also some pathogenic strains [31]. Unlike AMPs, however, lysozyme possesses enzymatic activity and causes bacterial lysis by hydrolyzing the peptidoglycan layer of the bacterial cell wall. Lysozyme was subsequently found in many animal tissues, physiological fluids of human, and in egg whites [32]. A few years later in 1928, Fleming discovered another antimicrobial substance called penicillin from the fungus *Penicillium notatum*. Penicillin is capable of killing a wide range of bacteria such as *Streptococcus*, *Meningococcus*, and *Bacillus*. It was the first conventional antibiotic to be discovered ever and marked the beginning of the golden era of antibiotics [33]. In the same year, Nisin, a polycyclic bacteriocin, was isolated from the bacteria *Lactococcus lactis* that displayed activity against Gram-positive bacteria, biofilms, and cancerous cells. The peptide binds to lipid II and exhibits its antibacterial activity by pore formation and by disrupting the cell division through lipid II extraction from the cell division site [34]. Almost 10 years later in 1939, Rene Dubos isolated gramicidin from the soil bacterium *Bacillus brevis*, the bacterium responsible for pneumococcal infection in mice [35, 36]. Nisin and gramicidin were the first peptide antibiotics to be discovered. Gramicidin was subsequently found to be a heterogeneous mixture of 6 *N*-formylated polypeptides with alternate L and D amino acids. Gramicidin is effective against a broad range of Gram-positive bacteria and could be used as an effective topical agent to treat wounds and ulcers on the skin. Gramicidin was the first AMP to be commercialized as an antibiotic [37]. Discovery of host defense peptides in microorganisms paved the way for finding new AMPs in other living forms. In 1942, Stuart and Harris discovered an AMP from the wheat flour that exhibits antimicrobial efficiency against a broad range of plant pathogens like *Pseudomonas solanacearum* and *Xanthomonas campestris*. It turned out to be a sulfur-rich protein with low molecular weight and was named purothionin; it belongs to the thionin family of AMPs in the plant kingdom [38]. Gradually, it became evident that bacteria are a great source of natural antimicrobials with varied structural features. In 1947, a group of cyclic non-ribosomal polypeptides called polymyxins was discovered from *Bacillus*.

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Polymyxins preferentially bind to lipopolysaccharides of Gram-negative bacteria and disrupt the membrane. They, however, have toxicity associated with them and are therefore used as the last resort drugs [39, 40]. Besides bacteria, insects also produce AMPs to protect themselves from invading pathogens. In 1967, an AMP called melittin was isolated from the venom of honeybee, *Apis mellifera.* Melittin eventually turned out to be a non-selective lytic peptide that displays activity against both Gram-positive and Gram-negative bacteria, viruses, parasites, mammalian cells, and cancer cells [41-43]. A few years later, the first AMP from amphibians called Bombinin was discovered from the skin of yellow-bellied toad *Bombina variegate*. It displays activity against both Gram-positive and Gram-negative bacteria as well as mammalian cells [44]. In 1973, researchers discovered the first group of cyclic peptides in plants and termed them the cyclotides; kalata B1 was the first cyclotide to be isolated from the African herb, *Oldenlandia affinis* [45]. The peptide displays activity against Gram-positive bacteria, viruses, and insects [46]. The peptide also possesses uterotonic properties. In 1980, Hans Boman and co-workers conducted a landmark study by introducing bacteria into giant silk moth, *Hyalophora cecropia* and isolating AMPs called P9A and P9B that exhibit activity against *E. coli* and other Gram-negative bacteria [47]. The sequences and structures of these peptides were subsequently determined, and they were renamed as cecropins [48]. This discovery in insects has led to the understanding of how insects protect themselves from microbes without an adaptive immune system. By then, many of the clinical strains had developed resistance against the miracle drugs, penicillin and streptomycin. Peptides were being looked at with great expectations as the next class of antimicrobial compounds. In 1983, Lehrer and coworkers identified two very similar microbicidal peptides, MCP-1 and MCP-2 from rabbit alveolar macrophages [49]. Each of these peptides harbors six cysteines involved in three disulfide linkages. They subsequently identified six more such peptides from rabbit granulocytes [50, 51]. In 1985, they reported three similar AMPs from human neutrophils and termed them human neutrophil peptides 1-3 (HNP 1-3). They also coined the term 'defensins' for these peptides [52, 53]. As another class of defensins was subsequently discovered, HNP 1-3 were termed as α-defensins. α-defensins are β-sheet rich peptides produced by neutrophils, natural killer cells, and monocytes; are effective against bacteria, viruses, and parasites; and possesses wound healing activity [52]. Another milestone in AMP research

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was achieved in 1987 when Michael Zasloff while operating on the African clawed frog, *Xenopus laevis* observed that the surgical wounds that he made in frogs abdomen healed without any inflammation or infection. This made him hypothesize that there must be some antimicrobial substance present in the frog skin that can protect the animal from infections. After a few months of this observation, Zasloff isolated two closely related peptides, magainin 1 and 2 from the frog skin [54]. Magainins are α- helical peptides with a broad range of antimicrobial activity. Iwanaga and coworkers isolated a 17-residue peptide, named tachyplesin from the hemocytes of the horseshoe crab, *Tachypleus tridentatus* in 1988 [55]. Tachyplesin has a β-hairpin structure stabilized by two inter-strand disulfide linkages and displays activity by forming pores in the bacterial membrane. Histatins, the histidine-rich antifungal peptides, were isolated from human parotid secretion in the same year [56]. Bovine tracheal antimicrobial peptide (TAP) was the first β-defensin to be isolated in 1991; it displays activity against Gram-positive and Gram-negative bacteria as well as fungus [57]. In the same year, another AMP PR 39 was isolated from pig intestine. It is highly rich in proline and arginine residues and active against both Gram-positive and Gram-negative bacteria [58]. Human cathelicidin LL-37 was isolated from immune cells in 1995 [59, 60]; it attains an α-helical conformation upon interaction with lipid membrane. Expression of LL-37 is transcriptionally regulated by vitamin D. Administration of vitamin D as supplement leads to the expression of LL-37 in epithelial cells which may prevent urinary tract infection in human [61]. In 1999, the first mammalian theta-defensin was discovered from the leukocytes of Rhesus macaque (*Macaca mulatta*) [62]. Rhesus theta defensin-1 (RTD-1) is a cyclic AMP produced by the ligation of two truncated α-defensins. The peptide takes an elongated structure stabilized by three disulfide linkages giving it a theta (θ)-like appearance. The peptide is active against Gram-positive and Gram-negative bacteria, viruses, and fungi [62]. Liu et al. identified a 47-residue peptide named LCI from *B. subtilis* that exhibits activity against plant pathogens, *Xanthomonas* and *Pseudomonas* [63]. Lu and coworkers determined the atomic resolution structure of the peptide in 2011 [64]. The peptide lacks cysteine and takes up a four-stranded antiparallel β-sheet structure. The peptide is unusually rich in aromatic amino acid residues (21%) and is highly thermostable. Jacob and coworkers have recently identified an AMP from the skin of south Indian frog *Hydrophylax bahuvistara*. The peptide

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was named urumin, and it acts against H1 hemagglutinin-bearing human influenza A viruses [65]. The literature reviewed here is quite concise, and a large number of peptides have been identified and isolated from natural sources.

1.4 Sequences and structures of antimicrobial peptides

Antimicrobial peptides are highly diverse in their sequence, amino acid composition, size, charge, and structure. This diversity makes it difficult to classify them, except by their three- dimensional conformations [66-69]. The major structural classes of AMPs are discussed here:

1.4.1 Alpha-helical peptides

Amphipathic α-helical peptides constitute the largest structural class of AMPs. Such peptides are found in almost all species ranging from invertebrates to higher organisms including human [13]. More than 400 α-helical AMPs are listed in the antimicrobial peptide database (APD3) as of today [23]. Amphipathic helices are generally less than 40 amino acid long with or without a hinge at the center and usually lack cysteine residues in their sequences [13]. These peptides are largely unstructured in water but tend to take an α-helical conformation in membrane-mimicking environments like trifluroethanol, hexafluoroisopropanol, sodium dodecyl sulfate micelles, and phospholipid vesicles [67, 70, 71]. Cecropins from insects [72], human cathelicidin LL-37 [73], and magainins from amphibians [74] are some of the well-studied peptides in this class (Figure 1.2). Melittin is yet another well-investigated α-helical peptide, but it displays largely indiscriminate permeabilization of lipid bilayers (Figure 1.2). Amphiphillic nature of mellitin has made it one of the most studied peptides for understanding peptide-membrane interactions [75]. This structural class includes some of the anionic AMPs as well *viz.* lysenin and dermcidin [76]. Some of the α-helical AMPs are listed in Table 1.1.

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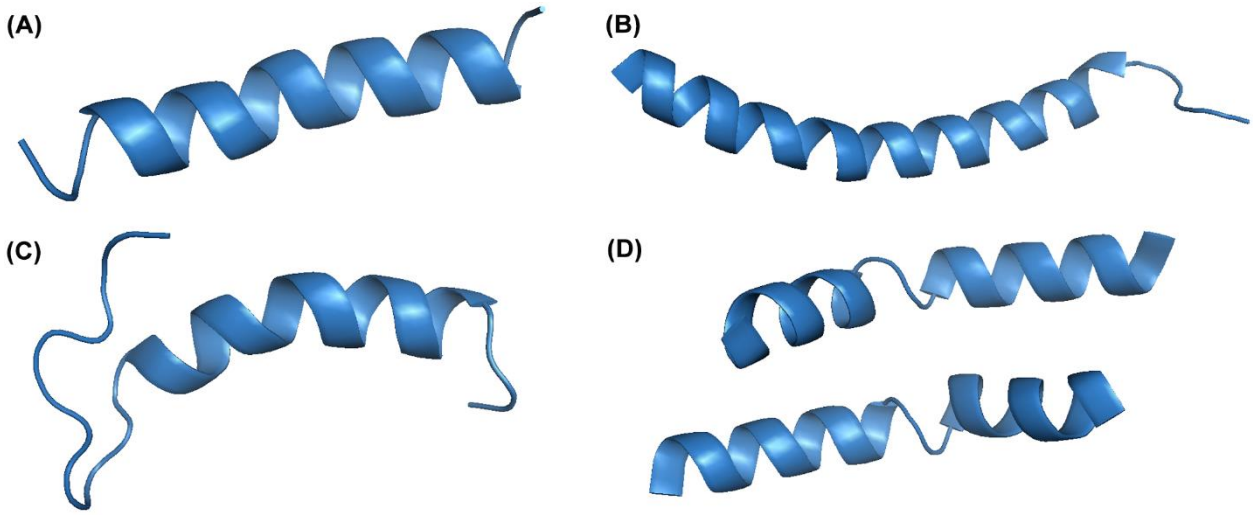

Figure 1.2 α-helical antimicrobial peptides. [A] magainin 2 (PDB ID: 2MAG), [B] LL-37 (PDB ID: 2K6O), [C] cecropin P1 (PDB ID: 2N92), and [D] melittin (PDB ID: 2MLT).

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Table 1.1 α-helical antimicrobial peptides [23]

Peptide	Source	Sequence	Length	Charge
Anoplin	*Anoplius samariensis*	GLLKRIKTLL	10	+4
Aurein 1.1	*Litoria aurea, Litoria raniformis*	GLFDIIKKIAESI	13	+1
Buforin II	*Bufo bufo gargarizans*	TRSSRAGLQFPVGRVHRLL RK	21	+6
Bombinin H2	*Bombina variegata*	IIGPVLGLVGSALGGLLKKI	20	+3
Cecropin A	*Hyalophora cecropia*	KWKLFKKIEKVGQNIRDGII KAGPAVAVVGQATQIAK	37	+7
Dermaseptin-B2	*Phyllomedusa bicolor*	GLWSKIKEVGKEAAKAAA KAAGKAALGAVSEAV	33	+4
Dermcidin	*Homo sapiens*	SSLLEKGLDGAKKAVGGLG KLGKDAVEDLESVGKGAV HDVKDVLDSV	47	-10
Histatin 5	*Homo sapiens*	DSHAKRHHGYKRKFHEKH HSHRGY	24	+12
Magainin 2	*Xenopus laevis*	GIGKFLHSAKKFGKAFVGEI MNS	23	+3
Melittin	*Apis mellifera*	GIGAVLKVLTTGLPALISWI KRKRQQ	26	+6
Maximin 4	*Bombina maxima*	GIGGVLLSAGKAALKGLAK VLAEKYAN	27	+4
Pleurocidin	*Pleuronectes americanus*	GWGSFFKKAAHVGKHVG KAALTHYL	25	+4
LL 37	*Homo sapiens*	LLGDFFRKSKEKIGKEFKRI VQRIKDFLRNLVPRTES	37	+6
Temporin A	*Rana temporaria*	FLPLIGRVLSGIL	13	+2
Pseudin-2	*Pseudis paradoxa*	GLNALKKVFQGIHEAIKLI NNHVQ	24	+2
Phylloseptin-1	*Phylllomedusa hypochondrialis*	FLSLIPHAINAVSAIAKHN	19	+2
Clavanin A	*Styela clava*	VFQFLGKIIHHVGNFVHGF SHVF	23	+5
Hylin a1	*Hypsiboas albopunctatus*	IFGAILPLALGALKNLIK	18	+3
Piscidin 1	*Morone saxatilis*	FFHHIFRGIVHVGKTIHRLV TG	22	+3

1.4.2 β-sheet peptides

Peptides that take up β-hairpin or β-sheet conformation comprise another important structural class of AMPs. Such peptides would typically harbor cysteine residues, and the structure is stabilized through one or more intramolecular disulfide linkages [77]. Largely

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due to the structural constraints put on them by disulfide linkages, these peptides generally remain ordered in both aqueous solution and membrane-mimicking environments [24]. Studies indicate that the number of disulfide bonds affects the structure as well as the activity of these AMPs [78, 79]. Some of the well-studied β-sheet peptides include lactoferricin with a single disulfide bond, protegrin with two disulfide bonds, and human α and β-defensins with three intramolecular disulfide bonds (Figure 1.3) [66, 80]. Some of the AMPs of this class are listed in Table 1.2.

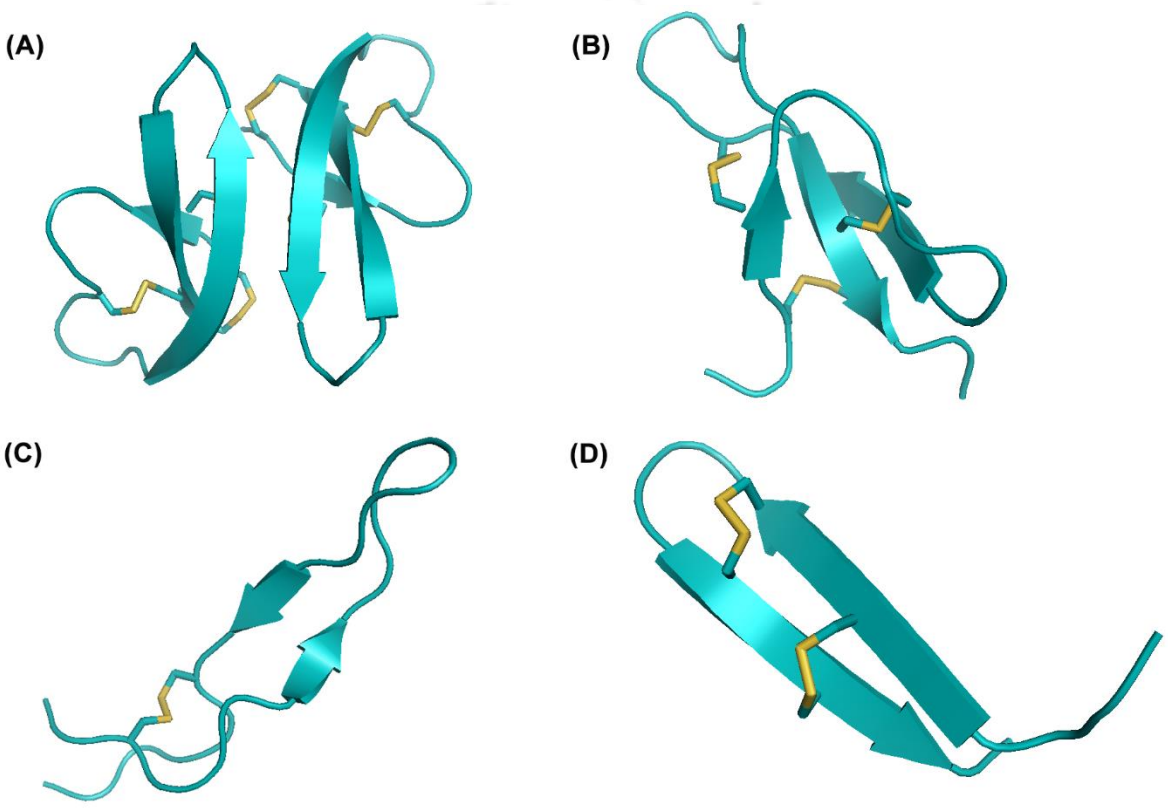

Figure 1.3 Antimicrobial peptides with β-sheet structure. [A] human α-defensin 1 (PDB ID: 3GNY), [B] Rabbit kidney defensin 1 (RK-1) (PDB ID:1EWS), [C] lactoferricin (PDB ID: 1LFC), and [D] protegrin 1 (PDB ID: 1PG1) [81-83].

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Table 1.2 List of selected β-sheet-rich AMPs [23]

Peptide	Source	Sequence	Length	Charge
LCI	*Bacillus subtilis*	AIKLVQSPNGNFAASFVLDGT KWIFKSKYYDSSKGYWVGIYE VWDRK	47	+3
Gramicidin S	*Bacillus brevis*	VKLFPVKLFP	10	+2
Microcin J25	*Escherichia coli* AY25	GGAGHVPEYFVGIGTPISFYG	21	-1
Lactoferricin B	*Bos taurus*	FKCRRWQWRMKKLGAPSITC VRRAF	25	+8
Thanatin	*Podisus maculiventris*	GSKKPVPIIYCNRRTGKCQRM	21	+6
Gomesin	*Acanthoscurria gomesiana*	QCRRLCYKQRCVTYCRGR	18	+6
Protegrin 1	*Sus scrofa*	RGGRLCYCRRRFCVCVGR	18	+7
Polyphemusin I	*Limulus polyphemus*	RRWCFRVCYRGFCYRKCR	18	+8
Tachyplesin I	*Tachypleus tridentatus*	KWCFRVCYRGICYRRCR	17	+7
Bovine neutrophil β-defensin 12	*Bos taurus*	GPLSCGRNGGVCIPIRCPVPM RQIGTCFGRPVKCCRSW	38	+6
Human neutrophil peptide-1	*Homo sapiens*	ACYCRIPACIAGERRYGTCIY QGRLWAFCC	30	+3
Hepcidin	*Homo sapiens*	ICIFCCGCCHRSKCGMCCKT	20	+3

1.4.3 Circular peptides

The end-to-end cyclized peptides constitute another structural class of AMPs. Circular AMPs have been discovered and characterized recently [84]. They are comprised of 14-70 amino acid residues. Due to their circular and stable structure, they are believed to be advantageous than the linear AMPs [84]. Bacteriocin AS-48 is the largest known circular protein isolated from *Enterococcus faecalis*. The three-dimensional structure is composed of five α-helices connected by five short turn regions that surround the hydrophobic core. Bacteriocin AS-48 is a highly stable peptide with a transition temperature (T_m) of 93.4 °C [85-87]. Besides bacteria, plants produce a group of cyclic peptides called cyclotides. Kalata B1 was the first cyclotide to be discovered. It is a 21-residue peptide, rich in cysteine, threonine, and glycine. Its three-dimensional structure comprises β-sheets with three disulfide bonds and a specific cysteine knot formed by two of the disulfide bonds [88]. These structural features render unusual stability so that peptides retain their biological

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activity by sustaining proteolytic degradation as well as high temperature [89]. Rhesus theta defensin (RTD 1) is an 18-residue cyclic peptide isolated from the leucocytes of rhesus macaque. Its three-dimensional structure consists of two β-strands connected by two turns and backbone stabilized by three disulfide bonds. RTD 1 is a highly cationic peptide due to the presence of five arginine residues and exhibits potent antimicrobial activity [62, 90]. Figure 1.4 shows some of the examples of cyclic AMPs.

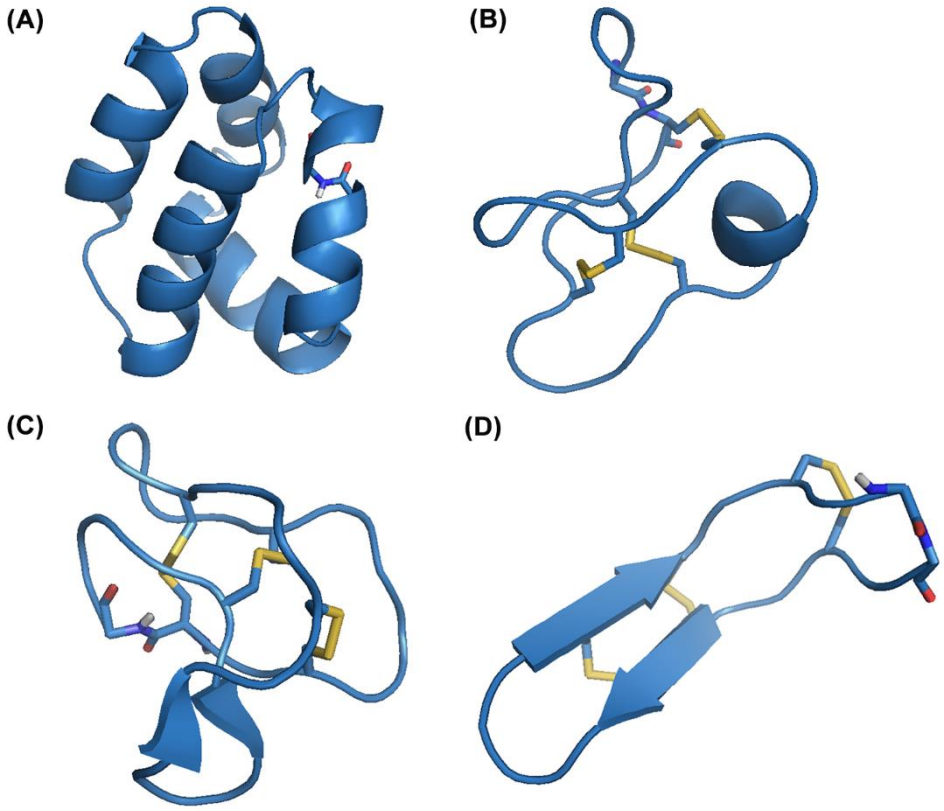

Figure 1.4 Cyclic antimicrobial peptides. [A] enterocin AS-48 (PDB ID: 1E68), [B] circulin A (PDB ID: 1BH4), [C] kalata B1 (PDB ID: 1KAL), [D] rhesus θ-defensin 1 (PDB ID: 1HVZ).

Besides backbone-cyclized peptides, few AMPs are cyclized through disulfide bridges. For example, polyphemusin I isolated from the horseshoe crab *Limulus polyphemus* and tachyplesin I isolated from horseshoe crab *Tachypleus tridentatus* are composed of antiparallel β-strands connected by a turn. These peptides are 17-18 residues long, amidated at C-terminus, and their structure stabilized by two disulfide bridges. Polyphemusin I and tachyplesin I are reported to have a broad spectrum of antimicrobial activity [91]. Figure 1.5 shows selected β-hairpin forming peptides.

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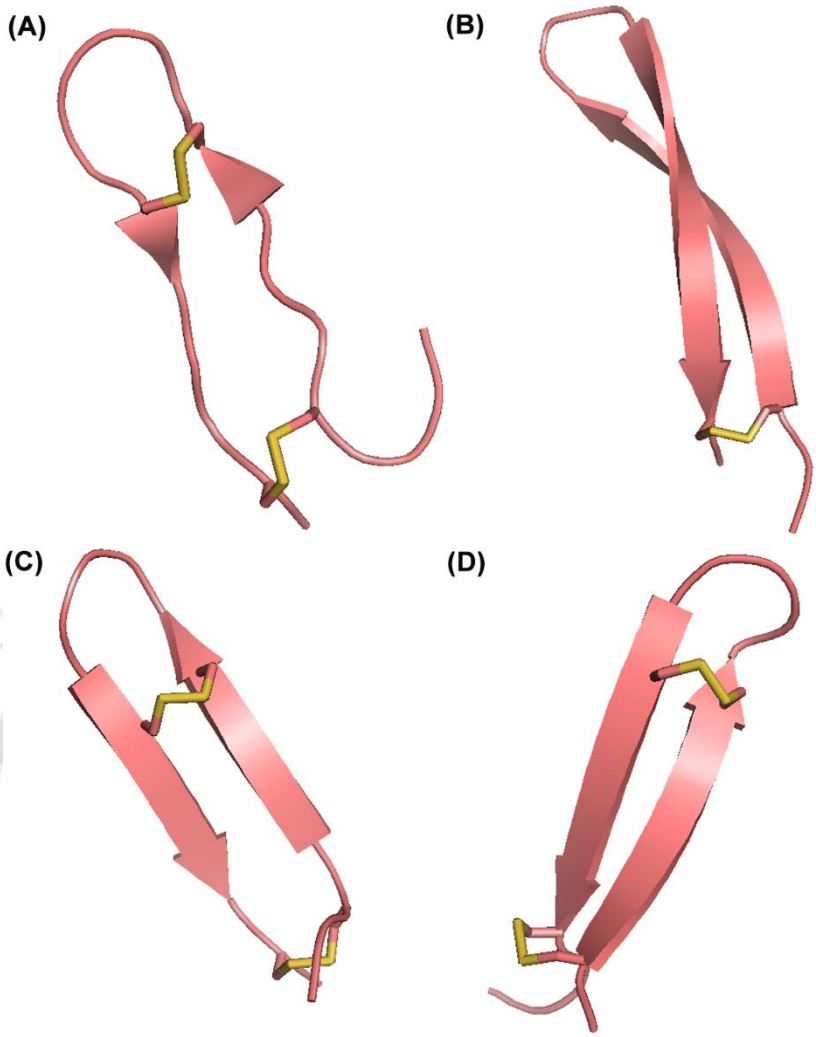

Figure 1.5 Cyclization by disulphide bridges. [A] polyphemusin I (PDB ID: 1RKK). [B] arenecin (PDB ID: 2JNI) [C] tachyplesin (PDB ID: 1WO1) [D] gomesin (PDB ID: 1KFP)

1.4.4 α-β structured peptides

The peptides made up of both α-helices and β-strands constitute another structural class (Figure 6). Human β-defensin 1 (HBD-1), for example, is a 36-residue peptide with a single helix and three β-strands linked by three disulfide bridges. HBD-1 is effective against Gram-positive and Gram-negative bacteria, cancer cells, and viruses [92].

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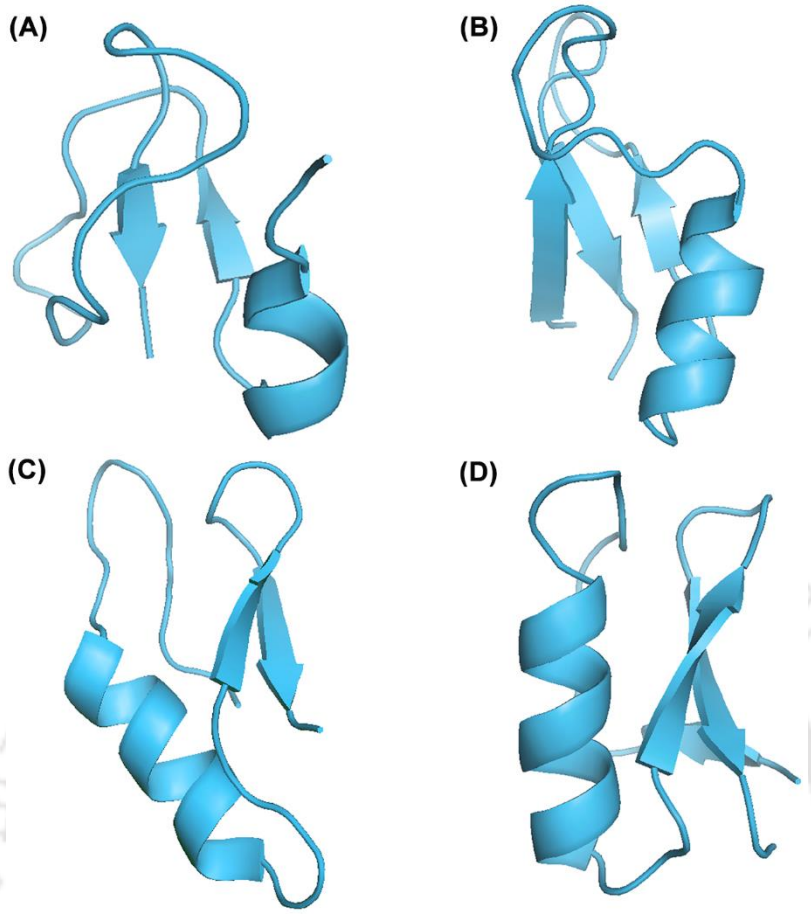

Figure 1.6 α-β structured antimicrobial peptides. [A] human β-defensin-1 (PDB ID: 1E4S), [B] heliomicin (PDB ID: 1I2U), [C] plectasin (PDB ID: 1ZFU), and [D] drosomycin (PDB ID: 1MYN). The peptides shown in the figure contain one or more disulfide bonds that are not shown for clarity.

Plectasin, the first fungal defensin isolated from saprophytic ascomycete *Pseudoplectania nigrella*, is active against *Streptococcus pneumonia* and resistant bacterial strains like MRSA [93]. The three-dimensional structure is composed of single α-helix and two β-strands stabilized by three disulphide bridges. Drosomycin is the first inducible antifungal protein isolated from insects, and its structure is composed of a single helix and twisted three-stranded β-sheet stabilized by four disulphide bridges [94]. It is active against filamentous fungi like *Aspergillus fumigatus*, *Aspergillus ustus* and *Fusarium solani* but inactive against bacteria. Heliomicin is another α-β antifungal peptide produced by lepidopteran, *Heliothis virescens*; the peptide folds into a structure made up of an alpha helix and a three-stranded β-sheet [95]. Few examples of α-β-structured AMPs are listed in Table 1.3.

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Table 1.3 Antimicrobial peptides with α-β structural motif

Peptide	Source	Sequence	Length	Charge
Coprisin	*Copris tripartitus.*	VTCDVLSFEAKGIAVNHSACALH CIALRKKGGSCQNGVCVCRN	43	+3
Cycloviolacin O14	*Viola odorata*	GSIPACGESCFKGKCYTPGCSCSK YPLCAKN	31	+3
HBD-1	*Homo sapiens*	DHYNCVSSGGQCLYSACPIFTKIQ GTCYRGKAKCCK	36	+4
Heliomicin	*Heliothis virescens*	DKLIGSCVWGAVNYTSDCNGEC KRRGYKGGHCGSFANVNCWCET	44	+2
Kalata B2	*Viola betonicifolia*	GLPVCGETCFGGTCNTPGCSCTW PICTRD	29	-1
Mytilin B	*Mytilus edulis*	SCASRCKGHCRARRCGYYVSVLY RGRCYCKCLRC	34	+9
Phormicin	*Phormia terranovae*	ATCDLLSGTGINHSACAAHCLLR GNRGGYCNGKGVCVCRN	40	+3
Plectasin	*Pseudoplectania nigrella*	GFGCNGPWDEDDMQCHNHCKS IKGYKGGYCAKGGFVCKCY	40	+1
pBD-2	*Sus scofa*	DHYICAKKGGTCNFSPCPLFNRIE GTCYSGKAKCCIR	37	+4
Termicin	*Pseudacanthotermes spiniger*	ACNFQSCWATCQAQHSIYFRRAF CDRSQCKCVFVRG	36	+5
Drosomycin	*Drosophila melanogaster*	DCLSGRYKGPCAVWDNETCRRV CKEEGRSSGHCSPSLKCWCEGC	44	+1

1.4.5 Non- α-β structured peptides

The non-α-β class of AMPs essentially includes the peptides that display activity without adopting any appreciable secondary structure. Nineteen such peptides are listed in the APD3 database, and they happen to be rich in one or more amino acids [23]. Members of this group include tryptophan-rich indolicidin and tritrpticin, and proline-rich drosocin and pyrrhocoricin. These peptides interact with the membrane through van der Waals interaction and H-bond in the extended conformation. Indolicidin happens to be most extensively studied peptide in this structural class (Figure 1.7). It is a 13-residue peptide isolated from bovine neutrophils. Out of these thirteen residues, five are tryptophans, and three are prolines [96]. The conformation of indolicidin is environment-dependent; it takes an extended conformation in the presence of negatively charged lipids but attains a bent conformation in the presence of zwitterionic lipids [97].

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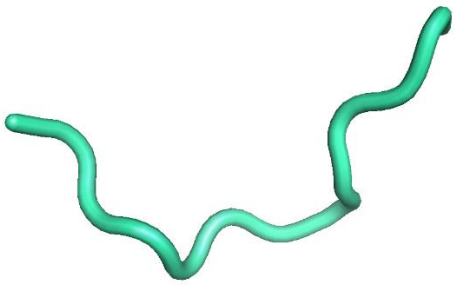

Figure 1.7 Extended structure of indolicidin (PDB ID: 1G89).

1.4.6 Peptides rich in specific amino acids

Peptides with unusually high content of one or more amino acids constitute yet another class of AMPs. PR-39, for example, is a 39-residue AMP having 19 prolines (~49%) and 10 arginines (~26%) [58]. Bactenecin, a 43-residue AMP, harbors 20 prolines (46.5%). Indolicidin is a 13-residue AMP with 5 Trp residues (~38%) while histatin-5 is a 24-residue peptide having 7 histidines (~29%) [98]. These peptides do not contain cysteine residues in their sequence and are generally linear in structure [66]. Some of the peptides of this class are listed in Table 1.4.

Table 1.4 Peptides rich in specific amino acids

Peptide	Source	Sequence	Length	Charge
Alloferon 1	*Calliphora vicina*	HGVSGHGQHGVHG	13	+4
Apidaecin IA	*Apis mellifera*	GNNRPVYIPQPRPPHPRI	18	+4
Astacidin 2	*Pacifastacus leniusculus*	RPRPNYRPRPIYRP	14	+6
Bactenecin	*Bos taurus*	RFRPPIRRPPIRPPFYPPFRPPIRPPI FPPIRPPFRPPLGPFP	43	+9
Heliocin	*Heliothis virescens*	QRFIHPTYRPPPQPRRPVIMRA	22	+5
Histatin-1	*Homo sapiens*	DSHEKRHHGYRRKFHEKHHSHR EFPFYGDYGSNYLYDN	38	+8
Histatin-5	*Homo sapiens*	DSHAKRHHGYKRKFHEKHHSHR GY	24	+12
Indolicidin	*Bos taurus*	ILPWKWPWWPWRRG	14	+3
Leptoglycin	*Leptodactylus pentadactylus*	GLLGGLLGPLLGGGGGGGGLL	22	0
Metchnikowin	*Drosophila melanogaster*	HRHQGPIFDTRPSPFNPNQPRPGP IY	26	+2
PR-39	*Sus scrofa*	RRRPRPPYLPRPRPPPFFPPRLPPRI PPGFPPRFPPRFP	39	+11

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1.4.7 Anionic peptides

Most natural AMPs are cationic; anionic peptides, however, do exist in nature and are composed of aspartic acid and glutamic acid residues that impart a net negative charge to the peptide [25]. Anionic AMPs are found in vertebrates, invertebrates, and plants as an indigenous component of their innate immune system [76]. They possess a net negative charge from -1 to -7 and their size ranges from 5-70 amino acid residues. They exhibit potent antimicrobial activity against both Gram-positive and Gram-negative bacteria. Human dermcidin and amphibian maximin H5 belong to this class of AMPs [99, 100].

1.5 Antimicrobial peptide databases

The rising discovery of AMPs since 20th century has led to a collection of large number of AMPs. Several AMP databases have been created in the past few years based on the sources, structures, and function of AMPs (Table 5).

Table 1.5 List of Antimicrobial peptide databases

Year	Database	Website	Content
1997	AMSDb	http://www.bbcm.univ.trieste.it/~tossi/amsdb.html (URL not active anymore)	Plant/animal AMPs
2002	SAPD	http://oma.terkko.helsinki.fi :8080/~SAPD (URL not active anymore)	Synthetic AMPs
2004	Peptaibol	http://www.cryst.bbk.ac.uk/peptaibol/home.shtml	Fungal AMPs
2004	APD	http://aps.unmc.edu/AP	Natural AMPs
2006	PenBase	http://penbase.immunaqua.com (URL not active as of now)	Shrimp AMPs
2006	Cybase	http://www.cybase.org.au/index.php	Cyclotides
2007	BACTIBASE	http://bactibase.pfba-lab-tun.org/main.php	Bacteriocins
2007	Defensins	http://defensins.bii.a-star.edu.sg	Defensins
2007	AMPer	http://www.cnbi2.com/cgi-bin/amp.pl (URL not active anymore)	Plant/animal AMPs
2008	RAPD	http://faculty.ist.unomaha.edu/chen/rapd/index.php (URL not active anymore)	Recombinant AMPs
2009	PhytAMP	http://phytamp.pfba-lab-tun.org	Plant AMPs
2010	CAMP	http://www.bicnirrh.res.in/antimicrobial	All AMPs
2014	DBAASP	https://dbaasp.org	Monomer, dimer, multi peptide
2016	DRAMP	http://dramp.cpu-bioinfor.org	All AMPs

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The first AMP database to be created was AMSDb. It contains around 45 antimicrobial proteins and 895 antimicrobial peptides from eukaryotes along with their precursor proteins, if any [101]. In 2004, another database called SAPD was created for synthetic antibiotic peptides [102]. Peptaibol database is a repository for sequences and structures of unusual peptides called peptaibols. Peptaibols are the peptides that harbor non-proteinogenic amino acids as well in their sequence. Some of the non-proteinogenic amino acids found in peptaibols include aminoisobutyric acid (Aib), isovaline (Iva), hydroxyproline (Hyp), and ethylnorvaline (Etnor). Peptaibols are synthesized by certain fungi such as *Trichoderma* and *Emericellopsis* species as secondary metabolites to ward off pathogens. N-terminus of petaibols is usually acetylated, and C-terminus is hydroxylated to an acid alcohol. Peptaibol repository consists of 317 sequences with 15-20 residues and contains information regarding the sequence, biological source, and 3D structures [103]. Alamethicin is one of the most-extensively studied peptaibol and forms voltage-dependent ion channels in membranes [104]. The antimicrobial peptide database (APD) which has been now upgraded to APD3 is a repository of natural AMPs. It comprises around 3000 AMPs from all the six kingdoms. It comprises the post-translationally-modified AMPs and some of the synthetic peptides as well. [23]. ANTIMIC was a general database that had a collection of AMPs from various sources but is not active anymore [105]. Two more databases were created in 2006; Penbase, that consists of peptides isolated from shrimps [106] and Cybase, that comprises naturally occurring cyclic peptides. Cyclic peptides are interesting as they are highly stable and could turn out to be promising candidates for next-generation antibiotics [107, 108]. Bactibase is a database of bacterial AMPs called bacteriocins produced by both Gram-positive and Gram-negative bacteria. It contains AMP data from 31 genera of bacteria wherein 156 peptides from Gram-positive bacteria and 18 from Gram-negative bacteria are listed [109]. As suggested by the name, the defensins knowledgebase database is a source of information related to defensins. At present, the database stores information about the structures, functions, and sequences of 363 defensins. The website also provides information about grants, clinical studies, and patents related to defensins [110]. AMPer is a database that uses information available in other databases to construct hidden Markov models to discover and develop novel AMPs [111]. RAPD is a database consisting information regarding recombinant approaches to develop

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AMPs. Data are collected from published experiments which includes information regarding protein fusion, expression, cleavage, and yield of the peptide [112]. PhytAMP is a dedicated repository for plant AMPs that provides information on taxonomy, structure, antimicrobial activity, target organism, and physiochemical properties of peptides [113]. $CAMP_{R3}$, an acronym for Collection of Anti-Microbial Peptides, release 3 harbors databases of sequences, structures, patents, and signatures [114]. The website also provides a tool named CAMPsign that allows identification of AMPs belonging to different AMP families. The database of antimicrobial activity and structure of peptides (DBAASP) consists of peptides for which antimicrobial activity against target organisms has been experimentally proved. It provides information regarding the peptide synthesis, whether it is ribosomal, nonribosomal, or synthetic. It allows the user to find information like the chemical structure of peptides, target organism, target entity, antimicrobial activity, hemolytic and cytotoxic effects [115]. Data repository of antimicrobial peptides (DRAMP) is the most recent addition to the AMP databases. It consists of 17611 AMPs that include 4833 general AMPs, 12704 patented AMPs, and 74 AMPs that are in clinical trials. This database is a great source of information regarding peptide sequences, their conformations, antimicrobial activity, physicochemical properties, patents, clinical trials, and references [4].

1.6 Mechanism of action

A drug molecule needs to come in contact with the target cell to exhibit its activity. The first encounter of AMPs takes place with the target cell surface. Bacterial cell surface is negatively charged largely due to the presence of anionic phospholipids that include phosphatidylglycerol, phosphatidylserine, and cardiolipin [26]. In addition to that, Gram-positive bacteria consist of lipoteichoic acid (LTA) as a major cell wall constituent. LTA are polymers of alternating sugar and phosphate groups linked through phosphodiester linkage. The phosphate groups contribute to the negative charge on the surface. The charge, however, can be neutralized if an amino acid modifies the sugars as is the case in type-I LTA. Teichoic acids play an important role in cell adhesion, cell shape determination, virulence, and antimicrobial resistance [116, 117]. The outer membrane of Gram-negative bacteria contains lipopolysachharide (LPS) which is essentially a lipid A

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molecule linked to complex carbohydrates that include anionic sugars [118]. The negative charge on LPS contributes to the negative charge on the Gram-negative bacteria. Eukaryotic membrane, on the other hand, is composed of neutral or zwitterionic lipids such as phosphatidylcholine, phosphatidylethanolamine, cholesterol, and sphingomyelin. Negatively charged lipids such as phosphatidylserine and phosphoinositides are present only on the inner leaflet on the membrane. [24]. Sterols like ergosterol and cholesterol stabilize the lipid bilayer and neutralize the AMPs [24, 119]. In addition to the difference in the net charge on the membrane, the difference in transmembrane potential between prokaryotes and eukaryotes is believed to contribute to the preferential binding of AMPs to the prokaryotic membrane. A mammalian cell membrane possesses a transmembrane potential of -90 to -110 mV whereas a bacterial cell in exponential growth phase has a transmembrane potential of about -130 to -150 mV [24]. Following membrane-interaction, AMPs can kill microbes in different ways. They can kill either by disrupting their membrane, by gaining access to intracellular targets and disturbing bacterial homeostasis, or both [120]. Membrane-disruption, as discussed above, happens to be the most common mechanism of cationic AMPs. In Gram-negative bacteria, cationic AMPs interact with negatively charged LPS present on the outer membrane. The peptide molecules then displace the divalent cations that support the membrane integrity by bridging the LPS moieties. Displacement of cations leads to destabilization of membrane, allowing AMPs to permeabilize the outer membrane by a process called self-promoted uptake [68]. Once the peptide molecules gain access to the inner membrane (cytoplasmic membrane), their modes of action branch off; membrane-disruptive peptides orient themselves and perturb the cytoplasmic membrane; non-membrane-disruptive peptides, on the other hand, translocate the membrane and target intracellular receptors [120].

1.6.1 Membrane-disruptive peptides

Three models that have been proposed to describe the membrane-disruption by AMPs are discussed below.

1.6.1.1 The barrel stave model

According to this model, the peptide molecules get aligned perpendicular to the membrane surface and form a barrel-like ring surrounding the aqueous pore. The hydrophobic region

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of the peptide interacts with the acyl chains of the lipids whereas hydrophilic region lines the aqueous pore [121-123]. Binding to the membrane surface, most likely as monomers, happens to be the first step. Following binding, the peptides undergo a conformational transition. Polar head groups are forced aside, and the hydrophobic region of the peptide interacts with the membrane. As soon as the peptide attains a threshold concentration in membrane, monomeric peptides tend to self-associate and insert deeper into the hydrophobic core of the membrane. Hydrophilic residues are less exposed to the hydrophobic core due to aggregation of peptides. With increasing peptide concentration, the pore size increases allowing the peptide molecules access to inner leaflet [24]. Such a mechanism is proposed for the fungal AMP, alamethicin that forms a channel with 8 peptide helices upon interaction with lipid bilayer [124].

1.6.1.2 The toroidal pore model

According to the toroidal pore model, the amphipathic peptides take up an α-helical conformation upon membrane interaction. The peptide molecules position themselves parallel to the surface of the membrane, and the hydrophobic region of the peptide displaces the polar lipid head group inducing a positive curvature-strain on the membrane creating a pore in the hydrophobic region [125]. As the peptide concentration increases, the membrane weakens and gets destabilized. At threshold concentration, the peptide molecules position themselves perpendicular to the membrane with the hydrophilic amino acids no longer exposed to the hydrophobic lipid chain [24]. This leads to the formation of a dynamic and transient peptide-lipid complex called the toroidal pore (Figure 10) [126]. The unique features of the toroidal pore include limited lifespan, discrete size, ion-selectivity, and requirement of optimum peptide charge. Magainin 2, for example, forms toroidal pores of ~2-3 nm in diameter thereby excluding the larger molecules [127, 128]. Moreover, the high charge on peptide results in intermolecular repulsion between the peptide chains causing destabilization and short life of the pores [119].

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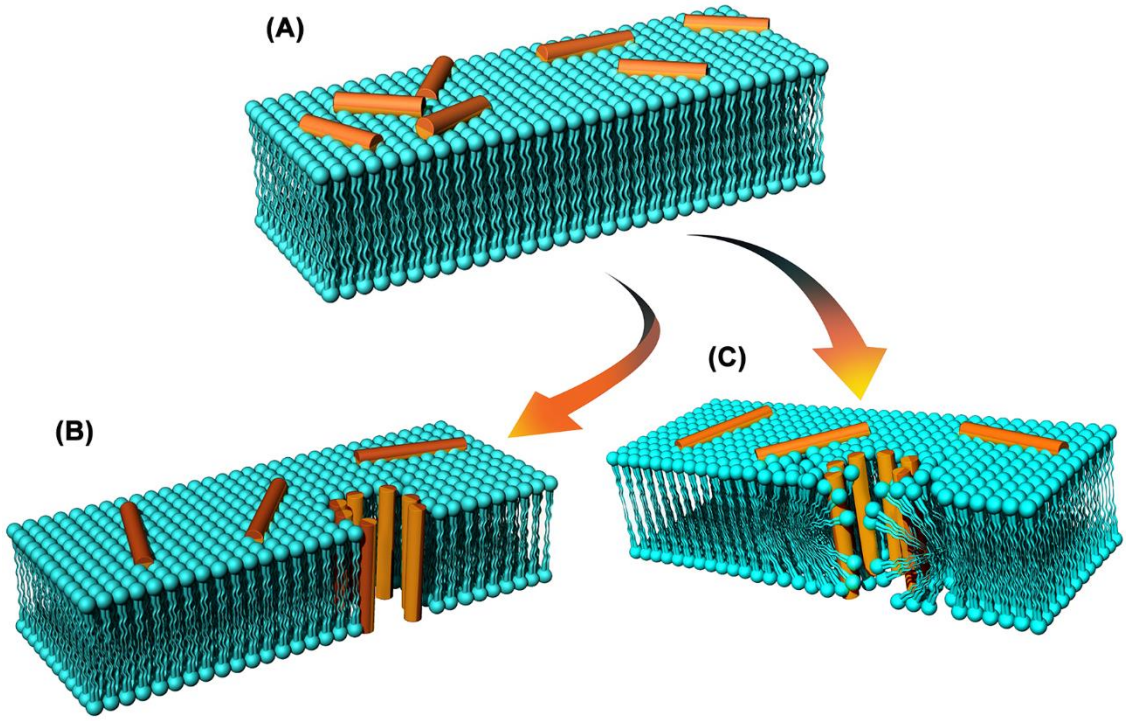

Figure 1.8 A diagrammatic representation of the barrel stave (panel B) and the toroidal pore (panel C) models.

1.6.1.3 The carpet model

The carpet model represents a nonspecific mode of AMP action. According to this model, the peptide molecules accumulate and align parallel on the membrane surface interacting with the lipid head groups. As the peptide concentration on the membrane surface increases and reaches a threshold concentration, membrane fluidity is compromised. Disruption of membrane potential ultimately leads to membrane disintegration [24, 120]. The carpet model is essentially a membrane disruption mechanism very similar to that of detergents [24]. Cecropin P1 is an example of AMP that disrupts the membrane though carpet mechanism [129].

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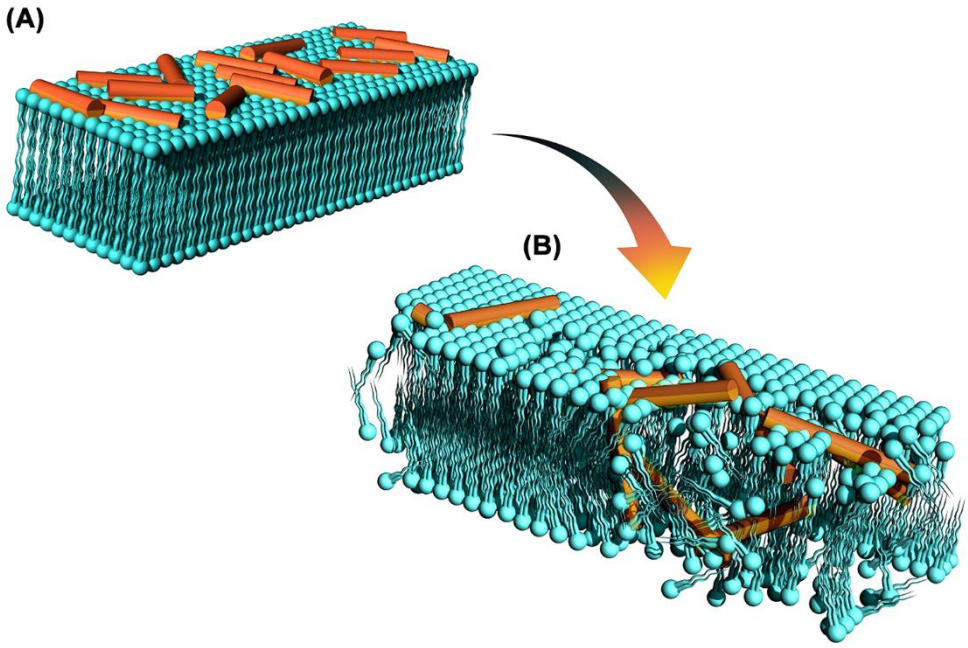

Figure 1.9 A diagrammatic representation of the carpet model. Panel A: binding of the peptides to the membrane, panel B: membrane-disruption.

1.6.2 Non-membrane-disruptive peptides

In the majority of cases, membrane perturbation is the key to antimicrobial peptide action. However, apart from interacting with the membrane, some AMPs translocate the membrane and target the intracellular processes such as protein and DNA synthesis [130]. PR-39, for example, inhibits protein synthesis. In addition to that, PR-39 induces degradation of some of the proteins essential for DNA replication [131]. Indolicidin, a potent AMP isolated from cytoplasmic granules of bovine neutrophils, is believed to translocate the membrane without disrupting it and targets intracellular receptors for bactericidal activity [132]. Insect AMPs like pyrrhocoricin, drosocin, and apidaecin cause cell death by inhibiting bacterial heat shock protein, DnaK [133]. Buforin II is a derivative of Buforin I, an AMP isolated from stomach tissue of Asian toad *Bufo garagriozans.* Buforin II is a potent antimicrobial agent against a broad range of organisms [134]. It takes up an amphipathic α-helical conformation in water:trifluoroethanol mixture as determined by NMR spectroscopy[135]. It, however, exerts antimicrobial activity without disrupting the membrane. The peptide is reported to bind to DNA and RNA after translocating the membrane and interrupt the cellular activities leading to cell death [132]. Microcin B17, a

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bacteriocin isolated from *E. coli* exhibit activity by targeting DNA gyrase [136]. Mersacidin, a lantibiotic from *Bacillus* binds lipid II and inhibits peptidoglycan biosynthesis. [137].

1.7 Selectivity of antimicrobial peptides towards Gram-positive or Gram-negative bacteria

Antibiotic-resistant microbes are at pinnacle globally. Common bacteria are resistant to many of the existing antibiotics. According to the World health organization's Global Antimicrobial Surveillance System (GLASS) 2018 reports, *Escherichia coli*, *Klebsiella pneumonia*, *Streptococcus pneumonia*, *Staphylococcus aureus*, *Salmonella spp.*, *Acinetobacter*, *Neisseria gonorrhoeae* and *Shigella* happen to be the most common drug-resistant bacteria. Majority of these are Gram-negative bacteria. Sadly, most of the drugs that have been developed in the last few decades are active against Gram-positive bacteria. Low efficiency of the drugs against Gram-negative bacteria is due to their complex membrane structure that prevents the entry of drug molecule across the membrane [138]. Therefore, strategies targeting the bacterial membrane itself could be the solution to this problem of drug's accessibility to the bacterial cytosol. AMPs with their unique features, therefore, can play an important role in treating Gram-negative bacterial infections. The peptides might also display synergistic effects with the conventional antibiotics. Lohner and Malanovic have recently analyzed the frog AMPs, the largest class of AMPs in AMP database, APD3. They observed that the peptides with a higher percentage of hydrophobic residues (61%-70%) display selectivity towards Gram-positive bacteria. Peptides with lesser hydrophobicity (41%-50%) are more active against Gram-negative bacteria [139]. However, the composition of bacterial membrane varies with species, making the selection process more complex. Designed amphipathic peptides with lower hydrophobicity need further investigation to validate this correlation between hydrophobicity with bacterial selectivity. Peptides with lower hydrophobicity and high amphipathicity can then be used to combat Gram-negative bacteria.

1.8 Immunomodulatory functions of antimicrobial peptides

Besides directly killing the pathogen, a majority of AMPs isolated till date are reported to have immunomodulatory activities as well [140]. They are reported to possess chemokine-

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like immunomodulatory activities that lead to a cascade of reactions thereby activating the adaptive immune responses [141-143]. Cathelicidins, for example, are secreted at the site of infection and induce the effector cells to produce chemokines and the mast cells to produce histamines thereby activating both innate and adaptive immune systems [144]. Cathelicidins from various organisms are chemotactic for all subsets of peripheral blood cells *in vitro* and *in vivo*. Human catehlicidin LL-37, for example, induces IL-8 release which in turn promotes the chemotaxis of neutrophils and release of high concentrations of LL-37 [143]. Human β-defensins also act as chemoattractants. HBD-1, which is produced constitutively, and HBD-2 and 3 that are induced upon infection are chemotactic for effector cells. HBD-2 brings dendritic cells and peripheral T cells (CD4+/CD45RO+) to the site of inflammation. The receptor used in this case is chemokine (C-C) receptor 6 (CCR6), which also recognizes the selective dendritic cell attractant macrophage inflammatory protein (MIP)-3α [1]. Human α-defensins directly attract immature dendritic cells derived from CD34+ progenitors or peripheral monocytes and also induces the migration of CD4+/CD45RA+ and CD8+ cells [145]. Besides higher animals, AMPs play crucial immunomodulatory roles in invertebrates as well. On invasion by pathogens, the circulating hemocytes travel to the site of injury due to chemotaxis and release AMPs. Peptides exert toxicity to the pathogen alongside inducing responses that control inflammation [146]. Host defense peptides also incite the release of specific cytokines; for example, limulus antilipopolysaccharide factor (LALF), an AMP isolated from the *Limulus polyphemus*, induces the release of antiviral and immunomodulating cytokines, IFN-α, IFN-γ, IL-2, and IL-13 from human peripheral blood mononuclear cells [144]. Experiments, wherein mice are infected with a lethal dose of *Pseudomonas aeruginosa*, have shown that LALF helps in survival by enhancing the synthesis of interleukins like IL-2, IL-12, and IL-13 [144, 147].

1.9 Antimicrobial resistance against antimicrobial peptides

Discovery of penicillin in 1928 revolutionized the field of medical research. It was the first antibiotic to be discovered in human history and marked the beginning of the "Golden era of antibiotics". Following the success of penicillin in treating life-threatening infections, Selman Waksman and his group discovered streptomycin in 1943, creating another

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landmark in the medical history. The advent of antibiotics brought a remarkable change in the expectancy and quality of lives. Antibiotics were regarded the miracle drugs and developed a notion that infections could be permanently defeated. Within a short span of time, however, the hope started dwindling as pathogenic bacteria started developing resistance against these drugs. The extensive use of antibiotics not only to treat infections but also used in animal husbandry, agriculture, and veterinary aggravated the condition [148, 149]. On top of that, the rate of development of new antibiotics reduced drastically to cope up with the crisis. Today, multidrug-resistant (MDR) organisms are posing a global threat and novel antimicrobials are desperately needed. Largely because of their mechanism of action, AMPs are gaining attention of researchers to be developed into next-generation antimicrobials. As AMPs kill through membrane permeabilization, the resistance against them is not prevalent. This is not to say that microbes cannot develop resistance against AMPs; AMP-resistant microbes are known [150]. Pathogens that confront AMPs mainly use two tactics to overcome them, intrinsic and acquired resistance. Intrinsic resistance occurs due to passive or inducible mechanism. In the passive mechanism of resistance, bacterial species like *Morganella*, *Providencia*, *Serratia*, and *Burkholderia* inherently produce more positively-charged lipid A that reduces the electrostatic interaction between the cell membrane and AMPs [151]. In the inducible mechanism, bacteria generally modify the cell membrane with cationic molecules on the cell surface. In Gram-positive bacteria, resistance against AMPs takes place through the incorporation of positively charged molecules into teichoic acid [152]. As soon as bacterial regulatory system senses the presence of AMP in the environment, it confers resistance by activating D-alanylation of teichoic acid. Bacteria like *S. aureus, S. pyogenes*, and *L. monocytogenes* carry out this modification to reduce the overall negative charge on their cell surface [153, 154]. In *S. aureus*, dltABCD operon system is responsible for D-alanylation of teichoic acid. Mutation in this operon leads to a higher negative charge on the cell surface; the bacterium, therefore becomes more susceptible to AMPs like α-defensins and cathelicidins [153]. Overexpression of these gene products, on the other hand, leads to increased D-alanylation and resistance to AMPs [153]. Besides, multiple peptide resistance factor MprF introduces L-lysine to the membrane phospholipids that reduces the net negative charge on the membrane, imparting resistance in *S. aureus* [155] and *B. anthracis*

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[156]. In Gram-negative bacteria, intrinsic resistance against AMPs is achieved by modifying the lipopolysaccharide [157]. Various regulatory systems like PmrAB, PhoPQ, and Rcs are involved in the resistance mechanism. To maintain a less negatively charged cell surface, bacterial regulatory system pmrCAB adds 4-aminoarabinose to lipid A; the modification lowers the binding affinity of AMPs [158]. The PhoPQ regulatory system is responsible for AMP resistance and virulence in *P. aeruginosa* [159]. PmrAB system regulates the modification of lipopolysaccharide in *Acenetobacter baumanni*[160]. Similarly, almEFG operon system in *Vibrio cholerae* facilitates the process of glycylation of lipopolysaccharides that confers resistance against polymyxins [161]. Many bacteria produce capsule to adhere to the tissues or to escape phagocytosis. Sometimes, this capsule acts as a shield to protect the bacteria from AMPs. Alginic acid, an anionic capsular exopolysaccharide produced by a virulent strain of *P. aeruginosa*, for example, is capable of diminishing the activity of cationic AMPs by limiting their access to the bacterial membrane [162].

One discernible way for bacteria to deactivate AMPs is to produce proteases and peptidases to degrade them. PgtE protein is one among the proteases that determine the resistance to AMPs through their degradation. PgtE is structurally similar to *E. coli* protease family OmpT [163]. These proteases cleave between basic amino acid residues and also at the carboxy-terminal of basic residues present before a nonpolar residue. For these features, the AMPs turn out to be the preferred targets of these proteases [24]. OmpT in *Salmonella* and *E. coli* have been reported to promote resistance against AMPs. *E. coli* grown in the presence of protamine, a salmon AMP, could counter the peptide activity through OmpT expression [164]. Furthermore, there are other proteases present in *E. coli* and *S. aureus* that confer resistance to AMPs. For example, heat shock serine protease DegP present in *E. coli* confers resistance against lactoferricin B [165].

Modifications such as myristoylation of LPS and acylation of lipid A in enterobacteria constitute another strategy to resist AMPs [166, 167]. Moreover, some organisms possess unusual component called phosphorylcholine (ChoP) on their cell surface that diminish the activity of AMPs [168]. Efflux is another common mechanism by which pathogens resist antimicrobials; thrombin-induced platelet microbicidal protein 1 (tPMP-1) from rabbit

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platelets is countered by certain Gram-positive bacteria like *S. aureus*. Studies revealed that the plasmid-encoded qacA gene helps the organism to eject the AMP out by a proton motive force efflux pump, and mutation in tPMP-1 gene reverses this effect [169].

AMP resistance can be transferred horizontally. A plasmid containing mcr-1 gene imparts resistance to *E. coli* against colistin through lipid A modification. Mcr-1 gene-harboring plasmid was initially found in commensal *E. coli* of livestock animals. The same mcr-1 gene was subsequently found in human isolates suggesting gene transfer [170, 171].

In addition to the structural changes in the cell membrane, AMP activity is also influenced by the energetics of the membrane. *S. aureus* with constitutive reduction of transmembrane potential is reported to cause resistance to some of the AMPs [24]. A similar phenomenon is reported in *Candida albicans* as well. Respiration-deficient *C. albicans* were produced by mutations in the mitochondrial DNA. The mutated strains were more resistant to the potent AMP, histatin-5 compared to the wild-type strain [172]. Furthermore, the activity of histatin-5 was reduced when wild-type strain was treated with inhibitors of the electron transport chain. These results suggested that the antimicrobial activity of histatin-5 is dependent on the energetics of the target cell.

1.10 Challenges in developing AMPs as antibiotics

Despite the identification and development of a large number of highly potent AMPs, the results of clinical trials are disheartening. Peptide-based drugs need to overcome certain challenges to be used as therapeutics [140]. Majority of the AMPs that have made up to the clinical trials are used in topical applications, rather than systemic application, indicating some toxicity associated with them. Even though the peptides preferentially bind to the negatively charged bacterial membranes, the large hydrophobicity of the peptides could lead to their interaction with host cells as well. Many AMPs cause lysis of erythrocytes to a considerable extent [173-175]. Polymyxins happen to be one of the most studied peptide drugs. Polymyxins kill bacteria by permeabilizing their inner and outer membranes. However, they also result in adverse neurotoxic and nephrotoxic effects which is why they are prescribed as the last resort drugs [176]. AMPs, therefore, need to be designed and optimized to minimize their toxicity to the host cells.

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Another property of natural AMPs that limits their use as therapeutics is their stability to the proteolytic enzymes; susceptibility to proteases underlies their poor pharmacokinetics [177]. Trypsin, for example, cleaves the peptides at the carboxyl side of lysine and arginine residues, the positive charge imparting residues in most AMPs [178]. AMPs, therefore, are considered as low oral-bioavailability drugs. In addition to that, if injected intravenously, AMPs are degraded within a short period by the enzymes present in the blood plasma and removed out of the body by the liver and the kidneys [179]. Incorporation of non-proteinogenic amino acids, such as D-amino acids and the amino acids disubstituted at α-carbon, in peptide sequences happens to be one of the strategies to increase the proteolytic stability of AMPs [180-183]. End-capping *i.e.* capping the peptide termini can impart stability from exopeptidases [177, 180, 184]. Moreover, improving the mode of drug delivery by entrapping the AMPs, in liposomes, for example, could increase their stability and reduce their toxicity towards host cells [185, 186].

As initial interaction of most AMPs with bacterial membranes is electrostatic in nature, salt is another factor that severely compromises their activity. The activity of HBD-1, for example, is hampered due to the high concentration of salt present in bronchopulmonary fluids in cystic fibrosis patients [187]. Similarly, P-113, a 12 amino acid fragment of histatin-5 displays reduced anticandidal activity in the presence of salts [188]. Substitution of tryptophan and histidine residues by bulkier non-natural amino acids, β-naphthylalanine and β-(4,4′-biphenyl)alanine has been shown to improve antimicrobial activity and reduce salt sensitivity in some peptides [189].

Another important concern in the development of AMPs as commercial drugs is their high cost of production. Large-scale production is a challenging task by solid phase chemical synthesis. The cost of synthetic peptide is almost five to twenty times higher compared to the conventional antibiotics [190]. Attempts are being made to find a solution to this problem; recombinant DNA technology is being used to express antimicrobial peptides in microbial systems [180]. Various production systems like bacterial, yeast, and mammalian cells are used to produce recombinant peptides. A variant of fungal defensin, plectasin, called AP114 was produced by recombinant technology to treat Gram-positive bacteria associated infections [93].

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1.11 Antimicrobial peptides as therapeutics

Largely due to their rapid killing and a broad spectrum of activity, AMPs are considered as the promising candidates for next-generation therapeutics. Among the thousands of AMPs discovered till date, however, only a handful has made it to the clinical trials. Polymyxin, the last resort drug discovered in 1950s has been approved for clinical use in the treatment of MDR Gram-negative bacteria [191]. Pexiganan, a 22-residue analog of magainin 2 is capable of disintegrating bacterial membrane and is administered as a topical cream for the infections related to diabetic foot ulcers. Sadly, however, it recently failed in the phase-III clinical trials [192]. Omiganan, a synthetic peptide derived from indolicidin kills both Gram-positive and Gram-negative bacteria as well as fungus [193, 194]. Omiganan is used as a topical gel. It is in phase III clinical trials and is being examined for catheter-related bloodstream infections and Rosacea. Lytixar (LTX-109), a synthetic peptidomimetic displays activity against a broad range of organisms including methicillin-resistant *S. aureus* [195]. LTX-109 is under phase II clinical trial and is used topically. Human lactoferrin-derived peptide, hLF1-11 is an antimicrobial and anti-inflammatory peptide derived from the N-terminal region of lactoferrin protein. The peptide is in phase II clinical trials and administered intravenously for the treatment of both bacterial and fungal infections during stem cell transplantation [196]. CZEN-002 is a synthetic antifungal peptide used against vaginal infections. It is also active against bacteria and inhibits replication of HIV-1 virus. The peptide is currently in phase IIb clinical trials [26]. LL-37 possesses antimicrobial, wound healing, and cell proliferation properties [197]. It is used for treating chronic wounds like venous leg ulcers [198]. LL-37 comes in Polyvinyl alcohol-based solution and is under phase I/II clinical trials. Novexatin NP213 is a potent synthetic antimicrobial agent developed by NovaBiotics. It is a cyclic, arginine-based heptamer with potent antifungal activity that is used for treating nail infections. The physicochemical properties of the molecule facilitate rapid penetration through the surface of the nail into the nail bed without getting into the systemic environment. It is applied as a topical brush treatment in the affected nail and is currently under phase II clinical trials [199]. Octo plus 145 (OP-145) is a 24-residue peptide derived from LL-37 that is used for the treatment of otitis media and is under phase II clinical trials [199]. *Clostridium difficile* infection (CDI) is the major cause of nosocomial diarrhea globally. NVB-302 is an antimicrobial developed

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from lantibiotics for the treatment of CDI and has successfully cleared phase I clinical trials. PXL01 is a synthetically-derived peptide from human lactoferrin. It is in phase II clinical trials and displays an inhibitory effect on scar formation by reducing infections, barring inflammation, and promoting fibrinolysis [200]. PXL01, formulated in sodium hyaluronate, is reported to reduce the formation of adhesions during surgical flexor tendon repair in hands without affecting the healing process [201]. A similar effect has been observed in rabbit model; PXL01 promotes the expression of a glycoprotein called lubricin which promotes the anti-adhesive activity [202]. Iseganan hydrochloride is the salt of synthetic protegrin and is presently in phase III clinical trials [203]. It is used for treating oral infections in myelosupressed cancer patients that undergo cytotoxic chemotherapy. Topical iseganan HCl efficiently eradicates oral aerobic bacteria and yeast infections by disrupting their cell membranes. Arenicin, avidocin, purocin, and IMX924 are other novel peptide-based drugs from various sources, currently under preclinical trials (Table 6) [199].

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Table 1.6 List of antimicrobial peptides in clinical trials [26, 199]

AMP	Description	Phase	Indication	Administration	Company
Omiganan	Synthetic cationic peptide derived from indolicidin	III	Catheter infections and Rosacea	Topical gel	BioWest Therapeutics/ Maruho
OP-145	Synthetic 24-mer peptide derived from LL-37 for binding to LPS or LTA	II	Chronic bacterial middle-ear infection		OctoPlus
Novexatin	Cyclic cationic peptide	IIb	Onychomycosis (Fungal infections of the toe nail)	Topical brush-on-treatment	NovaBiotics
Lytixar (LTX-109)	Synthetic, membrane-degrading peptide	I/II	Nasally colonized MRSA	Topical hydrogel	Lytix Biopharma
NVB302	Class B lantibiotic	I	*C. difficile* infection	Oral	Novacta
hLF1-11	Derived from lactoferricin (human)	II	Bacteremia and fungal infections in immunocompromised haematopoetic stem cell transplant recipients	Intravenous	AM-Pharma
CZEN-002	Dimeric octamer derived from α-MSH (human)	IIb	Vaginal candidiasis	Vaginal gel	Zengen
LL-37	LL-37 (human)	II	Hard-to-heal venous leg ulcers	Polyvinyl alcohol-based solution in the wound bed	Promore pharma
PXL01	Derived from lactoferricin (human)	III	Prevention of post-surgical adhesion formation in hand surgery	Hyaluronic acid-based hydrogel; administration at the surgical site	Promore pharma
Iseganan	Derived from protegrin (porcine leukocytes)	III	Oral mucositis in patients receiving radiotherapy for head and neck malignancy	Oral solution	IntraBiotics pharmaceuticals
PAC-113	Derived from histatin-3 (human saliva)	II	Oral candidiasis in HIV seropositive patients	Mouth rinse	PacGen
Arenicin	21 amino acids; rich in arginine and hydrophobic amino acids	Pre-clinical	Multidrug-resistant Gram-positive bacteria	–	Adenium Biotech
Avidocin and purocin	Modified R-type bacteriocins from *P. aeruginosa*	Pre-clinical	Narrow spectrum antibiotic for human health and food safety	–	AvidBiotics
IMX924	Synthetic 5-amino acid peptide, innate defense regulator	Pre-clinical	Gram-negative and Gram-positive bacteria	–	Iminex

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1.12 Hypothesis: membrane-binding stretches in microbial proteins can turn out to be potential AMPs

According to the AMP database APD3, ~8% of AMPs isolated till date are of bacterial origin [23]. Bacterial origin AMPs date back to 1939 when gramicidin, the first commercialized AMP, was discovered from *B. brevis* [35, 36]. Subsequently, more AMPs such as nisin, microcin, and daptomycin were discovered. Teixobactin, a recently discovered peptide-like antibiotic, is also of bacterial origin [204]. Bacteria, therefore, happen to be a great source of not only conventional antibiotics but AMPs as well. As most AMPs exhibit their activity through membrane-permeabilization, it would be interesting to investigate the antimicrobial properties of membrane-binding peptide stretches present in microbial proteins. We envisaged that such peptides, when employed from outside, can interact and muddle the bacterial membrane. Moreover, such self-like sequences can interfere with the intracellular activities of the native protein and can probably elude the degradation caused by intracellular proteases.

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भारतीय प्रौद्योगिकी संस्थान गुवाहाटी
Indian Institute of Technology Guwahati

CHAPTER 2

Materials and Methods

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2.1 Materials

NovaPEG Rink amide resin, fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids, and *N,N,N′,N′*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl) uronium hexafluorophosphate (HBTU) were purchased from Novabiochem (Darmstadt, Germany). *N,N*-diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA), ethanedithiol, thioanisole, piperidine, acetic anhydride, polymyxin B, 1-N-phenylnaphthylamine (NPN), 3,3'-dipropylthiadicarbocyanine iodide ($DiSC_3(5)$), α-cyano-4-hydroxycinnamic acid (HCCA), and sinapinic acid were from Sigma-Aldrich Chemicals Pvt. Ltd. 1-hydroxybenzotriazole hydrate (HOBt), diethyl ether, potassium chloride, ethylenediaminetetracetic acid potassium salt (EDTA), 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), and sodium dihydrogen phosphate (NaH_2PO_4) were from Sisco Research Laboratory, India. *N,N*-dimethylformamide (DMF) and *m*-cresol were purchased from Merck, India. 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), and cholesterol (CHL) were obtained from Avanti Polar Lipids. Bacterial and fungal growth media, nutrient media, Müller Hinton broth, Middlebrook 7H9 broth base, Middlebrook ADC growth supplement, RPMI 1640, yeast extract peptone dextrose (YPD), and triton-X 100 were obtained from HiMedia. The bacterial strains used were *Escherichia coli* (MG 1655), *Pseudomonas aeruginosa* (NCTC 6750), *Salmonella enterica* (SL 4213), *Xanthomonas oryzae* pv. *oryzae* (BXO43), *Staphylococcus aureus* (NCTC 8530), Gentamicin and Methicillin resistant *Staphylococcus aureus* (ATCC 33592), and *Mycobacterium smegmatis* (ATCC 607) while *Candida albicans* (ATCC 18804) was used for assaying antifungal activity.

2.2 Peptides

2.2.1 Peptide synthesis

Peptides were synthesized manually by solid phase peptide synthesis using Fmoc chemistry. All the peptides were synthesized on Rink amide resin using DMF as the solvent. Each amino acid was coupled twice wherein first coupling was carried out with 3-fold molar excess (3X) and second coupling carried out with 2-fold molar excess (2X) amino acid *w.r.t* the resin substitution (X). Protected amino acids were activated using

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equimolar HBTU, equimolar HOBt, and 2-fold molar excess of DIPEA. First coupling was carried out for minimum 60 minutes while second coupling was carried out for at least 30 minutes. Fmoc removal after amino acid attachments to the resin was carried out using 20% piperidine in DMF. The resins were washed scrupulously after each Fmoc removal step to ensure complete removal of piperidine. N-terminal acetylation of peptides, wherever applicable, was carried out on resin using 10-equivalents each of acetic anhydride and DIPEA. Synthesized peptides were cleaved from the resin using a cocktail mixture comprising TFA, ethanedithiol, thioanisole and *m*-cresol in a ratio of 20:1:2:2. Cleaved peptides were filtered through glass-wool and precipitated in glass tubes containing ice-cold diethyl ether. The precipitated peptides were subsequently washed using diethyl ether multiple times to ensure removal of chemical scavengers. The crude peptides were air-dried and stored at -20 °C.

2.2.2 Peptide purification and characterization

Peptides were purified on a reversed-phase C18 column using a linear gradient of water and acetonitrile (10-100%) containing 0.1% TFA on a Shimadzu Prominence Modular HPLC instrument. The collected peptide fractions were mixed with HCCA or sinapinic acid (10 mg/ml) matrix in the volume ratio of 1:2 (Peptide:matrix) and their identity ascertained using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry on a Bruker, Autoflex Speed MALDI TOF/TOF.

2.2.3 Peptide solutions and concentration estimation

Peptide stock solutions were prepared in water and their absorption spectra recorded from 210 – 350 nm. Concentrations of peptides that contained tryptophan and/or tyrosine residues were determined by taking the absorbance values at 280 nm and using Trp and Tyr molar absorption coefficients of 5690 $M^{-1}cm^{-1}$ and 1280 $M^{-1}cm^{-1}$, respectively. Concentrations of peptides that lacked Trp and Tyr but contained Phe were estimated by taking absorbance values at 254 nm and using a Phe molar absorption coefficient of 143 $M^{-1}cm^{-1}$ at 254 nm. Following concentration estimation, the peptides were diluted to the working stock solutions of 0.5 – 1 mM.

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2.3 Surface activity of peptides

Surface activity and membrane binding studies were carried out on a KSV Nima Langmuir instrument (Biolin Scientific) using a custom-made polytetrafluoroethylene trough of 13.2 cm^2 area. The trough contains a hole to allow addition of molecules into the subphase without disturbing the surface. Surface pressure was measured by Wilhelmy method using a platinum plate (Biolin Scientific). Phosphate buffer (10 mM, pH 7.4) was used as the aqueous subphase. Peptides were injected into the subphase and increase in surface pressure was monitored over time. Interaction of peptides with lipid monolayers was subsequently studied. POPC and POPG were used as the zwitterionic and negatively charged lipids, respectively. Lipid monolayers of POPC and 7:3 POPC:POPG were spread so as to obtain the surface pressures of ~30-32 mN/m. After stabilization of the surface pressure, peptides were gently injected into the subphase (final subphase peptide concentration =10 μM) through the hole in the trough without disturbing the monolayer. Mixing of peptides in subphase was achieved through gentle magnetic stirring. The change in surface pressure caused by the peptides was recorded as a function of time.

2.4 Preparation of small unilamellar vesicles

Small unilamellar vesicles (SUVs) were prepared with POPE:POPG (7:3) and POPC:CHL (10:1) lipid compositions. Lipids in the desired molar ratios were mixed and dried from stock solutions prepared in chloroform. Chloroform was evaporated using nitrogen gas to obtain thin lipid films. Dried lipids were desiccated for six hours and then hydrated overnight using 10 mM HEPES buffer, pH 7.4 containing 150 mM NaCl and 0.1 mM EDTA (HEPES-buffered saline, HBS) at room temperature. Following hydration, lipid suspension was vigorously vortexed and sonicated in a water bath sonicator till clear lipid suspensions were obtained [205].

2.5 Steady-state tryptophan fluorescence

Tryptophan fluorescence spectra were recorded for the peptides (1 μM) with and without SUVs in HBS. With SUVs, the spectra were recorded for 1:50 and 1:100 peptide:lipid molar ratios. The spectra were recorded on FP-8500 spectrofluorometer (Jasco) by exciting the samples at 280 nm for Tyr-lacking peptides and at 295 nm for Tyr-containing peptides.

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Emission spectra were recorded from 300 – 500 nm. Excitation and emission slit widths were 2.5 and 5 nm, respectively. The spectra were corrected by subtracting the corresponding blanks, *i.e.* samples without peptides.

2.6 Tryptophan fluorescence quenching

Peptide solutions (1 μM) in HBS, without and with SUVs (peptide:lipid ratio of 1:100) were excited at 295 nm and fluorescence emission spectra recorded from 300-500 nm. The samples were subsequently titrated with increasing amounts of acrylamide and fluorescence spectra recorded. Excitation and emission slit widths were 2.5 and 5 nm, respectively. The spectra were corrected by subtracting the spectra obtained from corresponding blanks *i.e.* the samples without peptides. Fluorescence intensity values at 350 nm were extracted and data were analyzed using Stern-Volmer equation, $F_0/F = 1 + K_{SV}[Q]$, where F_0 is the fluorescence intensity in the absence of quencher, F is the fluorescence intensity in the presence of quencher, K_{SV} is the Stern-Volmer constant, and [Q] is the quencher concentration [205]. K_{SV} value is an indicator of tryptophan accessibility to quencher; a larger K_{SV} value indicates higher accessibility. Inner filter effects were estimated with the formula $F = F_{obs}$ antilog$[(A_{ex} + A_{em})/2]$ where, F is the corrected fluorescence intensity and F_{obs} is the background subtracted fluorescence intensity of the sample. A_{ex} and A_{em} are the measured absorbance at the exitation and the emission wavelength [206].

2.7 Outer membrane permeabilization assay

The assay was carried out with *E. coli* cells using a non-polar probe, NPN. NPN is a fluorophore that weakly fluoresces in aqueous solutions, but exhibits enhanced fluorescence in hydrophobic environments. Perturbation of lipid bilayer allows NPN an access to the hydrophobic environment thereby causing an enhancement in its fluorescence. Cells were grown to mid-log phase, harvested, washed with 5 mM HEPES buffer, pH 7.4 having 5 mM glucose, and diluted to obtain an optical density of 0.5 at 600 nm. Subsequently, NPN was added to obtain a final concentration of 10 μM and fluorescence emission monitored at 420 nm by exciting at 350 nm. The excitation and emission slit widths were 2.5 and 5 nm, respectively. Peptides at different concentrations

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were added and fluorescence emission recorded as a function of time until no further increase in fluorescence is observed. Polymyxin B (10 μg/ml) was used as the positive control. Percentage NPN uptake is calculated using equation: % NPN = [(F_{obs} – F_0) / (F_{100} – F_0)] × 100, where F_{obs} is the observed fluorescence at a given peptide concentration, F_0 is the initial fluorescence of NPN with *E. coli* cells in the absence of peptide and F_{100} is the fluorescence of NPN with *E. coli* cells in the presence of 10 μg/ml polymyxin B [205].

2.8 Inner membrane permeabilization assay

Cytoplasmic membrane depolarization of *E. coli* cells was studied using $diSC_3(5)$ fluorescence. $DiSC_3(5)$ is a membrane-potential sensitive fluorophore. The dye partitions to the surface of polarized cells, gets concentrated and consequently self-quenched. Disruption of membrane potential releases the dye into the medium thereby causing an increase in its fluorescence. Mid-log phase bacterial cells in 5 mM HEPES buffer, pH 7.4 with 20 mM glucose were adjusted to an optical density of 0.05 at 600 nm. Subsequently, the cells were treated with EDTA (0.2 mM) for causing permeabilization of the outer membrane to facilitate the dye uptake. The cells were then treated with $diSC_3(5)$ such that the final dye concentration is 0.4 μM and incubated for 1 hour. Subsequently, KCl (100 mM) was added to equilibrate potassium ion concentration inside and outside the membrane. Fluorescence emission was recorded at 670 nm by exciting the sample at 622 nm. The excitation silt width was 1 or 2.5 nm, and emission slit width was 5 nm. Peptides were added at different concentrations and change in fluorescence intensity recorded as a function of time [205]. Polymyxin B (10 μg/ml) or 0.1% triton X-100 was used as the positive control for the assay.

2.9 Circular dichroism spectroscopy

Circular dichroism (CD) spectra were recorded on Jasco J-1500 spectropolarimeter in a 1 mm path length cell with a slit width of 1 nm and a step size of 0.2 nm. Spectra were recorded for peptides (50 μM) in trifluoroethanol (TFE) and in 10 mM phosphate buffer, pH 7.4 having 100 mM NaCl and 0.1 mM EDTA without and with POPC:CHL, and POPE:POPG SUVs (500 μM lipid concentration). Each spectrum is the average of 8 scans. The spectra are smoothened with Savitzky-Golay algorithm [207, 208] and presented as

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mean residual ellipticity, $[\theta]_{MRE}$. Mean residue ellipticity was calculated using the formula: $[\theta]_{MRE} = (M_r \times \theta_{mdeg})10 \times l \times c$, where Mr is the mean residue weight i.e. peptide molecular weight/number of residues, θmdeg is the ellipticity in millidegrees, l is the length in centimeters, and c is the peptide concentration in mg/ml [209].

2.10 Antibacterial assay

2.10.1 Colony count method

Mid-log phase cells were washed twice with 10 mM phosphate buffer, pH 7.4. The cells were then diluted in the same buffer to have approximately 10^6 colony forming units (CFU)/ml. One hundred microliters of the cell suspensions were treated with different concentrations of peptides (final volume adjusted to 120 µl) and incubated at 37 °C for 2 hours. Following incubation, the cell suspensions were 10-fold diluted in 10 mM phosphate buffer, pH 7.4 and 20 µL volumes were spread on nutrient agar plates or peptone sucrose agar plates (*Xanthomonas oryzae pv. oryzae*). Bacterial plates were incubated at 37 °C for 12-18 hours and for *Xanthomonas oryzae pv. oryzae* at 28 °C for 48 hours. The colonies were counted to determine the antimicrobial activity. The peptide concentrations that resulted in the complete killing of the microbes were considered the minimum lethal concentrations.

2.10.2 Broth microdilution method

Minimum inhibitory concentration (MIC) of the peptides were determined using broth microdilution method [210]. Fresh bacterial colonies were inoculated in Müller Hinton broth. Cultures were allowed to grow at 37 °C in a shaker incubator at 200 rpm to attain turbidity that is equal to McFarland standard 0.5 (0.08 - 0.1 OD at 625 nm). Cultures having higher turbidity were diluted with the media to obtain the desired turbidity. MIC was determined using broth microdilution method in polypropylene microtiter plates. Two-fold concentrated peptides were serially diluted from a range of 128 µM to 1 µM in microtiter wells; the final concentration of cultures in the wells was 5×10^5 CFU/ml. Growth control and sterility control were included for the reliability of the experiment. Microtiter plates were incubated at 37 °C for 16-20 hours, and turbidity was examined with

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unaided eyes. MIC is the lowest concentration of peptide that inhibits the growth of microbes resulting in clear wells in the microtiter plates.

2.10.3 Antimycobacterial assay

M. smegmatis culture was grown in Middlebrook 7H9 broth till it attained mid-log phase. Optical density (OD) of bacterial suspension was adjusted to 0.007 (2 X 10^5 CFU/ml) at 600 nm. Two-fold concentrated peptides were serially diluted from a range of 128 μM to 1 μM in microtiter wells (50 μl volume). 50 μl of the 0.007 OD cells were subsequently added to each well. Growth and sterility controls were included in the experiment. Microtitter plates were incubated at 37 °C for 48 hours, and turbidity was examined with unaided eyes. Concentrations at which no visible growth was observed was considered the MIC of the peptides.

2.11 Antifungal assay

2.11.1 Colony count method

Mid-log phase cells were washed twice with 10 mM phosphate buffer of pH 7.4. The cells were then diluted in the same buffer to have approximately 10^6 colony forming units (CFU)/mL. One hundred microliters of the cell suspensions were treated with different concentrations of the peptides (final volume adjusted to 120 μL) and incubated at 28 °C for 2 hours. Following incubation, the cell suspensions were 10-fold diluted in 10 mM phosphate buffer, pH 7.4 and 20 μL volumes were spread on yeast extract-peptone-dextrose (YPD) agar plates. Fungal plates were incubated at 28 °C for 24-30 hours. The colonies were counted to determine the antimicrobial activity. The peptide concentrations that resulted in the complete killing of the microbes were considered the minimum lethal concentrations.

2.11.2 Broth microdilution method

For yeast, MIC is determined by broth microdilution method as per Clinical and Laboratory Standards Institute (CLSI) M27-A3 manual. Briefly, 24 hour-old colonies were inoculated in 10 mM phosphate buffer, pH 7.4 and cell density adjusted to that of 0.5 McFarland standard (1 X 10^6 - 5 X 10^6 CFU/ml) at 530 nm. Cells were further diluted to 1 X

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10^3 - 0.5 X 10^4 CFU/ml in RPMI medium, buffered with 10 mM phosphate, pH 7.4 and supplemented with glutamine. Two-fold concentrated peptides were serially diluted in RPMI medium from a range of 128 μM to 1 μM in microtiter wells; followed by inoculation with culture (1:1 v/v ratio) achieving the desired final inoculum size of 0.5×10^3 - 2.5×10^5 CFU/ml. Microtiter plates were incubated at 30 °C for 24 hours and observed for the presence or absence of visible growth.

2.12 Salt sensitivity assay

2.12.1 Colony count method

Mid-log phase microbial cells were diluted in 10 mM phosphate buffer, pH 7.4, to obtain a cell concentration of 10^6 CFU/ml. Cultures were prepared in 100 mM NaCl or 2 mM $CaCl_2$ or 1 mM $MgCl_2$. Peptides at their lethal concentrations were added to 100 μL microbial suspensions (final volume adjusted to 120 μL) and incubated at 37 °C (bacteria) and 28 °C (fungus) for 2 hours. Aliquots of 20 μL were 10-fold diluted and spread-plated. Colonies were counted to determine the activity.

2.12.2 Broth microdilution method

For both bacteria and yeast, the sensitivity of peptides to 100 mM NaCl, 1 mM $MgCl_2$, and 2 mM $CaCl_2$ was ascertained by determining the MIC values in the presence of these salts. MICs were determined as described above (section 2.10.2, 2.10.3, and 2.11.2). A change in MIC indicates sensitivity to the salt.

2.13 Hemolytic assay

Human blood (~2 mL) was collected from a healthy individual in a tube containing ethylenediaminetetraacetic acid potassium salt and centrifuged at 800×g for 5 minutes. The pellet was resuspended in 5 mM HEPES buffer, pH 7.4 containing 150 mM NaCl. Erythrocytes were washed several times, and a 5% hematocrit was prepared in the same buffered saline. Peptides at various concentrations were incubated with 100 μL of 5% hematocrit and incubated for 1 hour at 37 °C. Following incubation, the hematocrit was centrifuged at 800 × g for 5 minutes and the absorbance of the supernatant was recorded at

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540 nm. Hematocrit incubated with deionized water was considered as the positive control (100% lysis).

2.14 FESEM analysis

Mid-log phase cells, bacteria and fungus (10^7) were treated with the minimum lethal concentrations of the peptides and incubated at 37 °C (bacteria) and 28 °C (fungus), respectively for 2 hours. Incubated cells were centrifuged at 1000×g for 5 minutes. The pellets were treated with 2.5% glutaraldehyde and incubated at 4 °C for 3 hours. Subsequently, cells were centrifuged at 1000×g for 5 minutes and the supernatant discarded. Cells were washed twice with 10 mM phosphate buffer, pH 7.4, loaded on glass slides, and dried at room temperature. The loaded samples were washed with deionized water and ethanol gradient ranging from 30%-100%. The samples were air-dried, sputter-coated with gold and analysed using FESEM.

2.15 Killing kinetics assay

Bacteria and yeast were treated with the minimum lethal concentration of the peptides. Aliquots of 20 µL from 10-fold diluted peptide-treated microbes were spread on agar plates after 1, 5, 15, 30, 60, and 120 minutes and antimicrobial activity was determined using methods similar to those described in the sections 2.10.1 and 2.11.1.

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CHAPTER 3

Antimicrobial Peptides from N-terminal Membrane-Binding Region of *E. coli* MreB Protein

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3.1 Summary

MreB is a bacterial cytoskeleton protein present in non-spherical cells localized beneath the cell membrane in the form of filaments [211]. MreB attributes to the survival of non-spherical bacteria by maintaining their cellular structure similar to the cytoskeletal elements like actin, tubulin, and intermediate filaments present in eukaryotic cells. MreB proteins exist in two different forms, one with a short amino acid stretch (~7-9 residues) at amino-terminus that could fold into an amphipathic α-helix and the other that lacks this N-terminal region. The N-terminal amphipathic helix in *E. coli* MreB is both essential and sufficient for its membrane binding [211]. I selected the 9-residue long N-terminal membrane-binding region of *E. coli* MreB protein and analyzed its membrane-binding propensity using Heliquest, a web-server that predicts the membrane-seeking potential of helices. The peptide was predicted to be a membrane-seeking one and subsequently investigated for its antimicrobial properties. The 9-residue peptide (C-terminal amide) and its N-terminal acetylated analog displayed broad-spectrum activity, killing Gram-negative bacteria, Gram-positive bacteria, and fungi. Extension with a tryptophan residue at the N-terminus drastically improved the activity of the peptides with lethal concentrations ≤10 μM against all the organisms tested. Tryptophan has been found in a majority of naturally occurring antimicrobial peptides. Several studies reported that introduction of a tryptophan moiety increases antimicrobial efficacy of the peptides as tryptophan efficiently interacts with the interfacial region of the membrane and facilitates peptide's partitioning through the lipid bilayer [212]. Studies suggest that hydrophobic amino acids at the N-terminus are required for antimicrobial activity. End-tagging of peptide with hydrophobic amino acids increases antimicrobial potency even in the presence of salts and serum [213, 214]. The tryptophan-extended peptides caused complete killing of *C. albicans* as well as gentamicin and methicillin resistant *S. aureus* at 5 μM concentration. Lipid-binding studies and electron microscopic analyses of the peptide-treated microbes suggest membrane disruption as the mechanism of killing.

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3.2 Results

3.2.1 Peptides

The helical-wheel projection of the 9-residue peptide stretch (MLKKFRGMF) from *E. coli* is shown in figure 3.1.

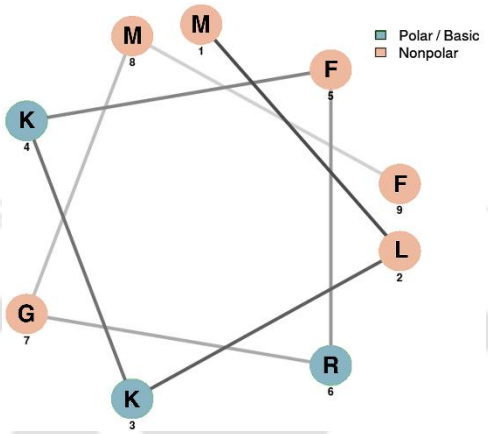

Figure 3.1 Helical wheel representation of MreB$_{1-9}$ sequence

Four peptides based on *E. coli* MreB$_{1-9}$ were designed and synthesized (Table 3.1). Mean hydrophobicity (<H>) and mean hydrophobic helical moments (<μH>) were calculated using Heliquest web server [215]. A discrimination factor (D) is defined as 0.944 <μH> + 0.33(z) to identify the possible lipid-binding helices. If the discrimination factor is higher than 1.34, the helix is predicted to be a membrane-binding one.

Table 3.1 Sequences and physicochemical properties of the peptides

Peptide name	Peptide sequence[a]	z[b]	<H>[c]	<μH>[c]	D
MreB$_{1-9}$	MLKKFRGMF-am	+4	0.528	0.762	2.039
Ac-MreB$_{1-9}$	Ac-MLKKFRGMF-am	+3	0.528	0.762	1.709
W-MreB$_{1-9}$	WMLKKFRGMF-am	+4	0.7	0.523	1.814
Ac-W-MreB$_{1-9}$	Ac-WMLKKFRGMF-am	+3	0.7	0.523	1.484

[a] 'Ac-' at N-terminus represents acetylated amino-terminus, while '-am' at C-terminus represents C-terminal amide.
[b] Net charge on the peptide at pH 7.4
[c] <H> and <μH> were calculated using Heliquest web server [215].

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As per the Heliquest discrimination factor criteria, all the four peptides qualified to be the lipid-binding ones. The peptides were synthesized, purified using reversed-phase HPLC (Figure 3.2), and their identities ascertained using MALDI-TOF mass spectrometry (Figure 3.3).

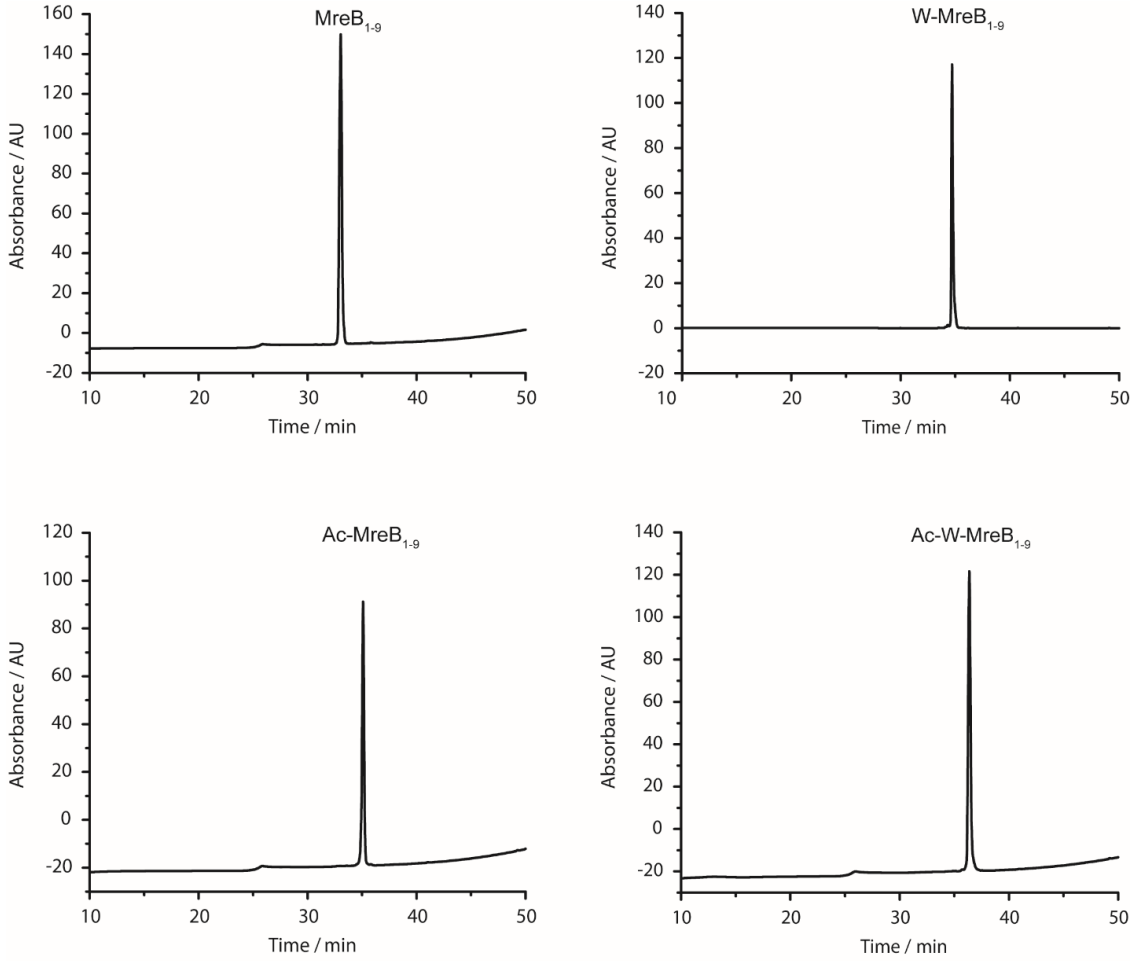

Figure 3.2 Reversed-phase HPLC chromatograms of the purified MreB-derived peptides

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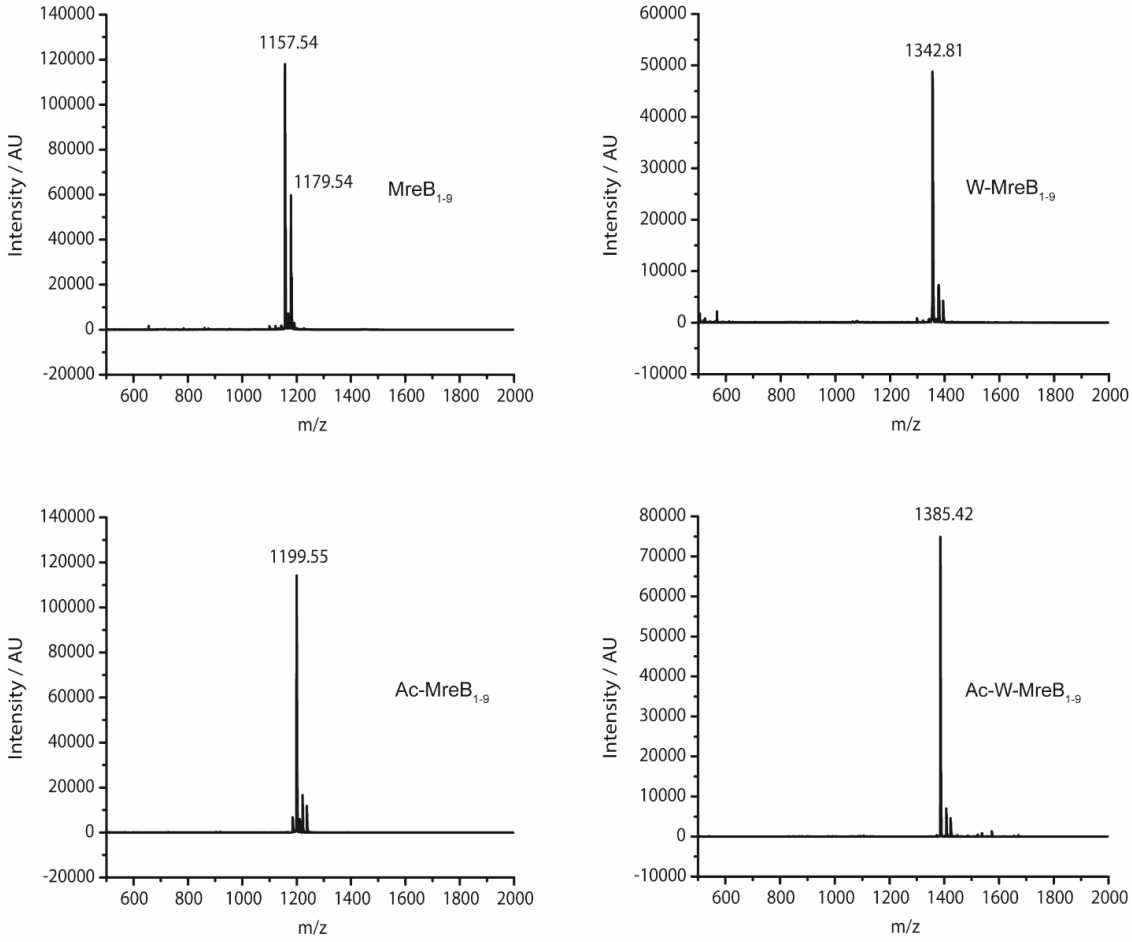

Figure 3.3 MALDI-TOF mass spectra of the purified MreB-derived peptides. The calculated monoisotopic masses for $MreB_{1-9}$, Ac-$MreB_{1-9}$, W-$MreB_{1-9}$, and Ac-W-$MreB_{1-9}$ are 1155.63 Da, 1197.64 Da, 1341.71 Da, and 1383.72 Da, respectively. The additional peak in the $MreB_{1-9}$ MALDI mass spectrum with m/z of 1179.54 Da corresponds to sodium adduct.

3.2.2 Surface activity and membrane binding of the peptides

Surface activity of peptides was measured using 10 mM phosphate buffer, pH 7.4 as the aqueous subphase. Peptides were injected into the subphase and changes in surface pressure monitored by the Wilhelmy method using a platinum plate. All the peptides caused substantial enhancement in surface pressure (≥5 mN/m) even at 2 μM peptide concentration (Figure 3.4).

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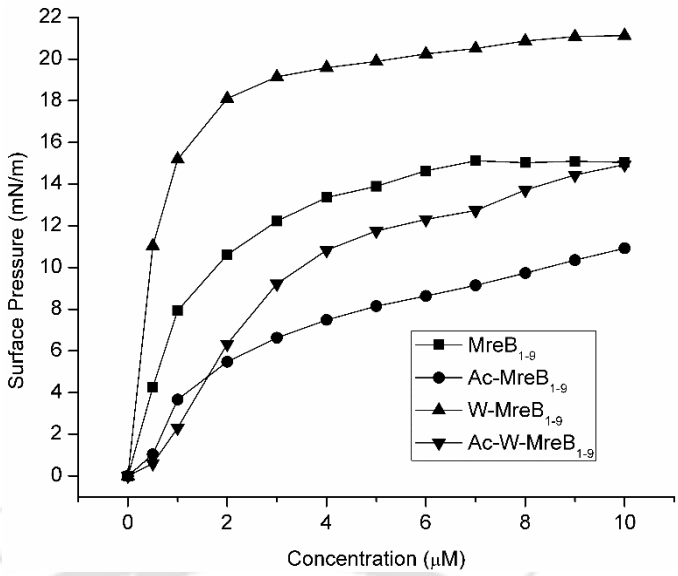

Figure 3.4 Surface activity of the MreB-derived peptides. Peptides at concentrations ranging from 0.5-10 μM were injected into the subphase. The stabilized surface pressure values are plotted against the peptide concentration.

The change in surface pressure, by and large, is higher for the peptides with higher net charge. Among the peptides with identical net charge, the longer peptides displayed larger increase in surface pressure. Interaction of peptides with lipids was studied using lipid monolayers. 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) were used as the zwitterionic and negatively charged lipids, respectively. Lipid monolayers of POPC and 7:3 POPC:POPG were prepared with surface pressures ~30-32 mN/m. Without disturbing the lipid monolayers, the peptides were injected into the subphase so as to have 10 μM concentration. The peptides caused ~2-6 mN/m increase in surface pressure for the POPC monolayer (Figure 3.5, Table 3.2)

Ac-W-MreB$_{1-9}$ caused 5.8 mN/m increase in the surface pressure while ≤3.6 mN/m change was observed for the other three peptides (Table 3.2). With POPC:POPG monolayers, all four peptides caused large enhancement in the surface pressure. Ac-W-MreB$_{1-9}$ caused 10 mN/m increase in surface pressure compared to 5.8 mN/m increase caused to POPC monolayers. Other three peptides caused ~8.5 mN/m increase to POPC:POPG surface pressure compared to ≤3.6 mN/m increase caused to POPC monolayers.

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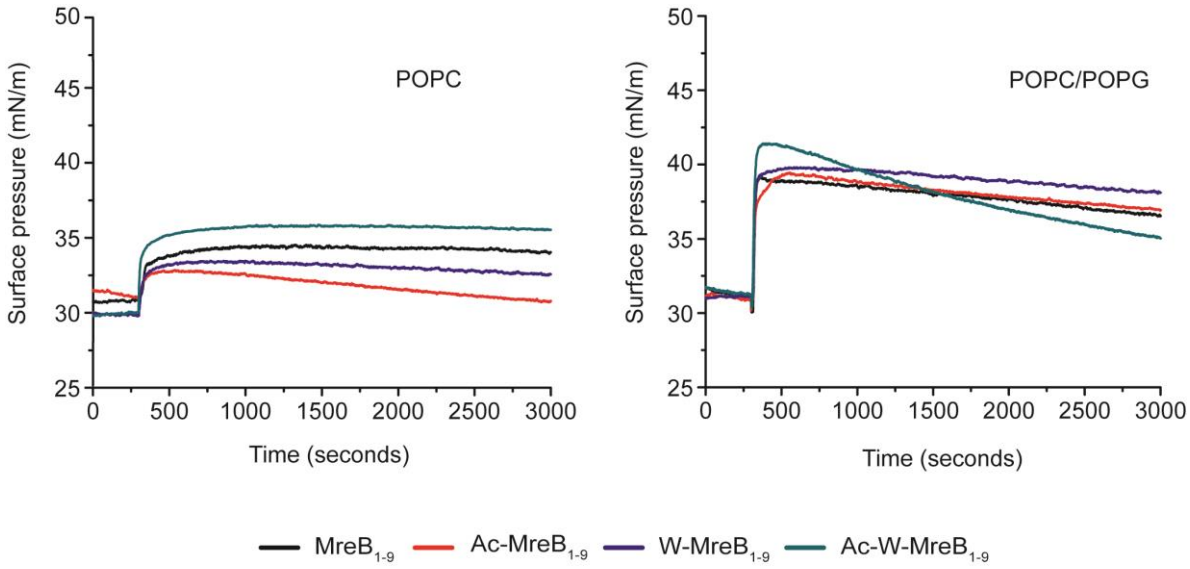

Figure 3.5 Increase in surface pressure caused by the MreB-derived peptides in POPC and POPC:POPG lipid monolayers.

Table 3.2. Maximum increase obtained in the surface pressure of the lipid monolayers on peptide addition (10 μM initial subphase concentration).

Lipid monolayer	Increase in surface pressure (mN/m)			
	$MreB_{1-9}$	$Ac\text{-}MreB_{1-9}$	$W\text{-}MreB_{1-9}$	$Ac\text{-}W\text{-}MreB_{1-9}$
POPC	3.6	1.8	3.6	5.8
POPC:POPG (7:3)	8.3	8.5	8.7	10

As predicted, all the peptides turned out to be the membrane-binding ones. The data suggest that the peptides preferentially bind to the negatively charged lipids as expected from the cationic, amphipathic peptides.

3.2.3 Tryptophan fluorescence

Binding of $W\text{-}MreB_{1-9}$ and $Ac\text{-}W\text{-}MreB_{1-9}$ to liposomes was studied using tryptophan fluorescence. In 10 mM HEPES buffer, pH 7.4 containing 150 mM NaCl and 0.1 mM EDTA (HEPES-buffered saline, HBS), the peptides display fluorescence emission band centered around 353 nm suggesting solvent-exposed tryptophan side-chain (Figure 3.6). In the presence of POPC:CHL SUVs, both the peptides display emission maxima ~346-348 nm at 1:100 peptide/lipid ratio. The little blue shift in the emission spectra suggests binding to liposomes. In the presence of POPE:POPG SUVs, $Ac\text{-}W\text{-}MreB_{1-9}$ displays emission maxima

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~345 nm and ~343 nm for peptide:lipid ratios of 1:50 and 1:100, respectively. W-MreB$_{1-9}$, on the other hand displays emission maximum ~340 nm at both the peptide:lipid ratios. Large blue shift observed for W-MreB$_{1-9}$ with negatively charged vesicles could be attributed to the charged amino-terminus. The data suggest preferential binding towards negatively charged membranes for both the peptides.

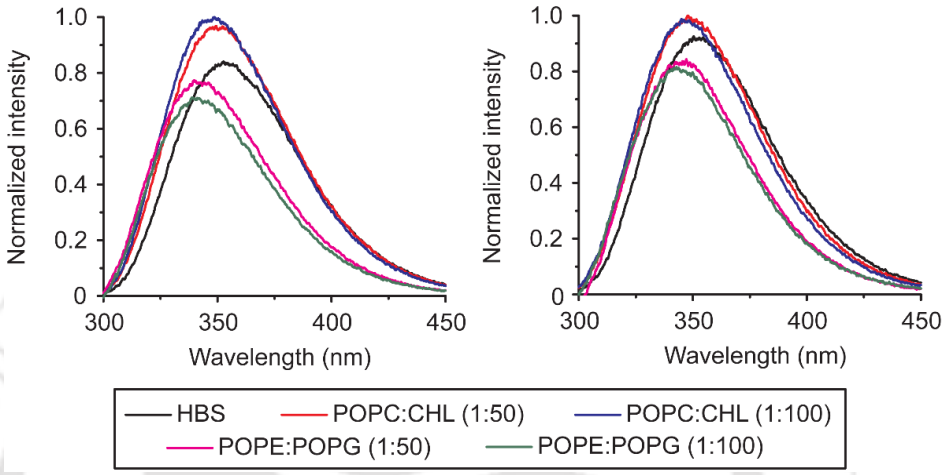

Figure 3.6 Trp fluorescence emission spectra of W-MreB$_{1-9}$ (left panel) and Ac-W-MreB$_{1-9}$ (right panel). The spectra were recorded in the absence of lipid (black trace) and in the presence of SUVS such that the peptide/lipid ratios are 1:50 and 1:100.

3.2.4 Tryptophan fluorescence quenching

Accessibility of tryptophan to the dynamic quencher, acrylamide was examined. Peptides (1 μM) in HBS, without SUVs and with SUVs (peptide:lipid ratio of 1:100) were titrated with increasing amounts of acrylamide and fluorescence emission spectra recorded as described in section 2.6, chapter 2. Stern-Volmer plots for W-MreB$_{1-9}$ and Ac-W-MreB$_{1-9}$ in the absence and presence of lipid vesicles were obtained using fluorescence emission intensities at 350 nm (Figure 3.7). Tryptophan side chains display high solvent accessibility for both the peptides in HBS which is in agreement with the emission maxima data in Figure 3.6. For the peptides in HBS, the Stern-Volmer plots deviate from linearity at concentrations higher than 0.06 M acrylamide. The Stern-Volmer constants, K_{SV} for the peptides in HBS were, therefore calculated using the linear region, *i.e.* from 0-0.06 M acrylamide concentration range. Stern-Volmer plots for the peptides in the presence of SUVs fit linearly and the K_{SV} values determined are shown in Table 3.3. The extent of

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quenching is considerably less in the presence of SUVs. The net accessibility factors (NAF) were calculated using the formula, NAF = K_{SV} (with SUVs)/K_{SV} (without SUVs).

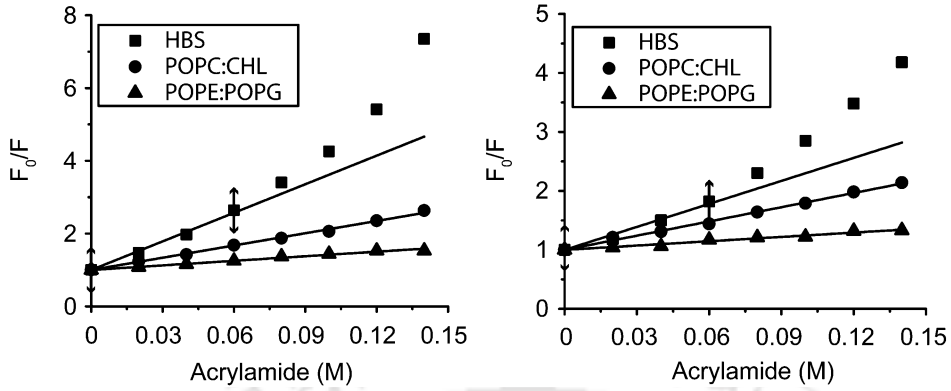

Figure 3.7 Stern-Volmer plots showing the tryptophan fluorescence quenching by acrylamide in W-MreB$_{1-9}$ (left panel) and Ac-W-MreB$_{1-9}$ (right panel) peptides in HBS (■), POPC:CHL SUVs(●), and POPE:POPG SUVs (▲). The vertical arrows indicate the concentration range of acrylamide used for fitting the data obtained in HBS.

Table 3.3 K_{sv} and NAF values of the MreB-derived peptides obtained from the quenching data shown in figure 3.7

	HBS		POPC:CHL		POPE:POPG	
	K_{SV} (M^{-1})	NAF	K_{SV} (M^{-1})	NAF	K_{SV} (M^{-1})	NAF
W-MreB$_{1-9}$	26.2	1	11.2	0.43	4.2	0.16
Ac-W-MreB$_{1-9}$	13.0	1	8.0	0.61	2.4	0.18

The data show that the tryptophan side-chain is largely inaccessible to the quencher in the presence of negatively charged lipid vesicles which indicates the presence of tryptophan residue inside a hydrophobic environment.

3.2.5 Antimicrobial assay

Antimicrobial activity of the peptides was investigated against Gram-positive and Gram-negative bacteria as well as fungus. The assays were carried out with the mid-log phase cells in 10 mM phosphate buffer, pH 7.4 as described in sections 2.10.1 and 2.11.1, chapter 2. Minimum lethal concentrations of the peptides are shown in table 3.4

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Table 3.4 Antimicrobial activity of the peptides

Microbe	Minimum Lethal Concentration (μM)[a]			
	$MreB_{1-9}$	Ac-$MreB_{1-9}$	W-$MreB_{1-9}$	Ac-W-$MreB_{1-9}$
Escherichia coli	20	15	5	10
Pseudomonas aeruginosa	10	100	3	10
Salmonella enterica	10	15	3	2
Staphylococcus aureus	50	100	10	10
Gentamicin and methicillin-resistant *Staphylococcus aureus*	20	20	5	5
Candida albicans	15	15	5	5

[a] Minimum peptide concentration that resulted in complete killing of the bacteria/fungus

$MreB_{1-9}$, the native sequence, possesses a net charge of +4 and exhibits high antimicrobial activity against both Gram-positive and Gram-negative bacteria as well as *C. albicans*. All the microorganisms tested, other than *S. aureus*, could be efficiently killed at 20 μM or lesser peptide concentration. The lethal concentration for *S. aureus* was 50 μM. Gentamicin-methicillin-resistant *S. aureus* (gentamicin-resistant MRSA), however, appeared more susceptible (lethal $MreB_{1-9}$ concentration = 20 μM). Capping of N-terminus with acetyl group renders the peptide less effective, particularly against *P. aeruginosa* and *S. aureus*. Interestingly, the activity against *C. albicans* and gentamicin-resistant MRSA is not affected on N-terminal acetylation. Extension of $MreB_{1-9}$ by a tryptophan residue at N-terminus makes the peptide (W-$MreB_{1-9}$) at least 3-times more active. All the microbes tested were efficiently killed at ≤10 μM peptide concentration. Bacterial membranes are negatively charged and most cationic amphipathic AMPs kill the bacteria by disrupting their membranes.

3.2.6 Salt sensitivity

The peptides retained substantial activity in the presence of 100 mM NaCl (Table 3.5). All four peptides displayed >75% killing of all the bacteria at their lethal concentrations. The activity against *C. albicans*, however, reduced to 38-54%.

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Table 3.5 Antimicrobial activity (percentage killing) of the MreB-derived peptides at their lethal concentrations in the presence of 100 mM NaCl, 1 mM $MgCl_2$, and 2 mM $CaCl_2$

Microbe → **Peptide ↓**		***E. coli***	***P. aeruginosa***	***S. enterica***	***S. aureus***	***Gentamicin-resistant MRSA***	***C. albicans***
$MreB_{1-9}$	NaCl	99.7±0.4	90.0±13.9	96.6±2.7	98.9±1.3	96.7±2.7	38.1±5.9
	$MgCl_2$	53±9.6	76.2±8.1	79.1±1.0	90.8±9.2	98.8±0.7	98.0±1.6
	$CaCl_2$	69.5±1.4	99.7±0.4	52.4±10.8	98.0±2.1	95.6±4.1	99.6±0.2
Ac-$MreB_{1-9}$	NaCl	98.6±1.9	97.8±1.4	78.6±11.0	99.6±0.6	93.2±7.7	54.0±9.6
	$MgCl_2$	0	42.0±5.8	65.6±6.7	93.6±9.2	97.2±3.6	98.5±1.0
	$CaCl_2$	4.6±3.4	100	2.5±1.1	97.4±3.9	96.4±1.8	57.0±6.1
W-$MreB_{1-9}$	NaCl	99.7±0.4	93.0±9.7	98.2±0.1	87.6±3.0	96.8±3.5	45.7±8.8
	$MgCl_2$	0	15.1±4.1	72.7±9.4	91.7±7.8	97.9±2.7	96.8±3.2
	$CaCl_2$	10.4±3.2	99.8±0.1	99.5±0.2	74.5±2.9	95.8±4.8	58.3±12.0
Ac-W-$MreB_{1-9}$	NaCl	100	100	100	93.5±9.7	92.0±8.8	43.4±8.9
	$MgCl_2$	100	100	96.7±3.8	79.9±4.9	95.7±3.0	99.9±0.1
	$CaCl_2$	100	100	100	83.5±1.4	89.6±3.3	92.5±8.8

Antibacterial activity of Ac-W-$MreB_{1-9}$ was little affected by the presence of salt showing complete killing of *E. coli*, *P. aeruginosa*, and *S. enterica;* and >90% killing of *S. aureus*. Activity against *C. albicans*, however reduced to ~43%. Divalent cations showed varied effects on the activity of peptides. $MreB_{1-9}$ retained >50% activity against all the microorganisms tested. The activity of Ac-$MreB_{1-9}$, on the other hand, was severely compromised against *E. coli* and *S. enterica* in the presence of divalent cations. Both $MreB_{1-9}$ and Ac-$MreB_{1-9}$ retained >90% activity against *S. aureus* in the presence of salt as well as divalent cations. Tryptophan-extended peptides exhibited better activity than the native sequences. The activity of W-$MreB_{1-9}$ against *E. coli* and *P. aeruginosa*, however, got severely compromised in the presence of Mg^{2+} ions. Ac-W-$MreB_{1-9}$, on the other hand, retained ~80% or more activity against all the four bacteria as well as fungus in the presence of divalent cations. $MreB_{1-9}$ is the sequence derived from *E. coli* and it is interesting to see that the native MreB peptide, $MreB_{1-9}$ retains >50% activity against *E. coli* in the presence of salt and divalent cations. The varied response to the divalent cations suggests that the peptides

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could work synergistically to achieve broad-spectrum killing under physiological conditions.

3.2.7 Outer membrane permeabilization assay

Permeabilization of *E. coli* outer membrane was studied using NPN fluorescence as described in section 2.7, chapter 2. Polymyxin B, a well-known outer membrane permeabilizing agent, was used as a positive control in the assay. Table 3.6 shows outer membrane permeabilizing ability of the MreB-derived peptides relative to polymyxin B.

Table 3.6 Outer membrane permeabilization of *E. coli* cells by MreB-derived peptides. The percentage NPN uptake was determined relative to that caused by 10 μg/ml polymyxin B.

Peptide concentration (μM)	$MreB_{1-9}$	Ac-$MreB_{1-9}$	W-$MreB_{1-9}$	Ac- W-$MreB_{1-9}$
	Percentage NPN uptake			
5	93.06±9.41	52.08±0.93	82.83±0.7	83.16±17.7
10	100	79.14±18.05	100	100
15	100	97.31±3.8	100	100

All the four peptides cause permeabilization of *E. coli* outer membrane. The maximal NPN uptake (~97%) for Ac-$MreB_{1-9}$ was obtained at 15 μM peptide concentration. For other three peptides, 100% uptake could be obtained at 10 μM concentration.

3.2.8 Inner membrane permeabilization assay

The assay was carried out using voltage-sensitive dye, $diSC_3(5)$ as described in section 2.8. Fluorescence emission from $diSC_3(5)$-treated *E. coli* cells was recorded at 670 nm after exciting the samples at 622 nm (slit width = 2.5 nm). Treatment of *E. coli* with $MreB_{1-9}$ and Ac-$MreB_{1-9}$ did not cause any appreciable enhancement in fluorescence (Figure 3.8). Rather, the fluorescence intensity was dramatically reduced for $MreB_{1-9}$. This decrease could be attributed to Outer membrane permeabilization that allows $diSC_3(5)$ ready access to the inner membrane. At 15 μM and 20 μM concentrations, however, the decrease in fluorescence is followed by a small increase, suggesting disruption of membrane potential. W-$MreB_{1-9}$ and Ac-W-$MreB_{1-9}$, on the other hand, rapidly permeablize the inner membrane causing enhancement in fluorescence intensity.

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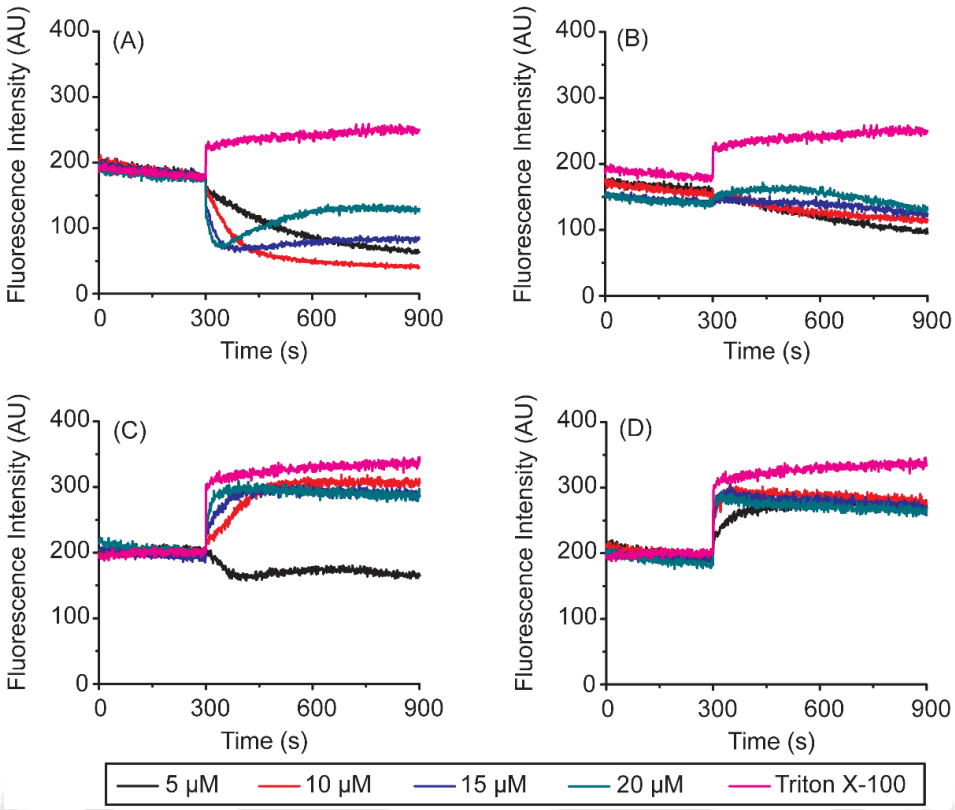

Figure 3.8 Inner membrane depolarization of *E. coli* cells by the MreB-derived peptides. An increase in fluorescence intensity of voltage-sensitive dye $diSC_3(5)$ following peptide treatment suggests depolarization of the inner membrane. Panels A, B, C, and D correspond to $MreB_{1\text{-}9}$, Ac-$MreB_{1\text{-}9}$, W-$MreB_{1\text{-}9}$, and Ac-W-$MreB_{1\text{-}9}$, respectively.

3.2.9 Circular dichroism spectroscopy

CD spectroscopic analysis shows that the peptides are unordered in 10 mM phosphate buffer, pH 7.4 having 100 mM NaCl and 0.1 mM EDTA (Figure 3.9). In the presence of POPC:CHL SUVs, W-$MreB_{1\text{-}9}$ becomes structured while other three peptides are largely unordered (panel C). All the four peptides show positive ellipticity ~195-197 nm in the presence of POPE:POPG SUVs, suggesting folding of the peptides. The spectrum for $MreB_{1\text{-}9}$ in the presence of POPE:POPG vesicles show negative bands around 222 and 205 nm and a weak positive band ~197. The spectrum is suggestive of a mixture of α-helical and random coil conformations (panel A). Ac-$MreB_{1\text{-}9}$ and W-$MreB_{1\text{-}9}$ fold into distinct α-helical conformation in the presence of negatively charged SUVs (panels B and C). Ac-W-$MreB_{1\text{-}9}$ shows a negative band centered ~226 nm and a positive band centered ~195 nm suggesting a mixture of α-helical and β-strand conformations. The data suggest that the peptides are unstructured in water but fold upon binding to negatively charged lipid

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vesicles. In TFE, MreB$_{1-9}$ spectrum suggests a mixture of α-helical and random coil conformations (panel A). Ac-MreB1-9, W-MreB$_{1-9}$, and Ac-W-MreB$_{1-9}$, on the other hand, take up distinct α-helical conformation (panels B-D).

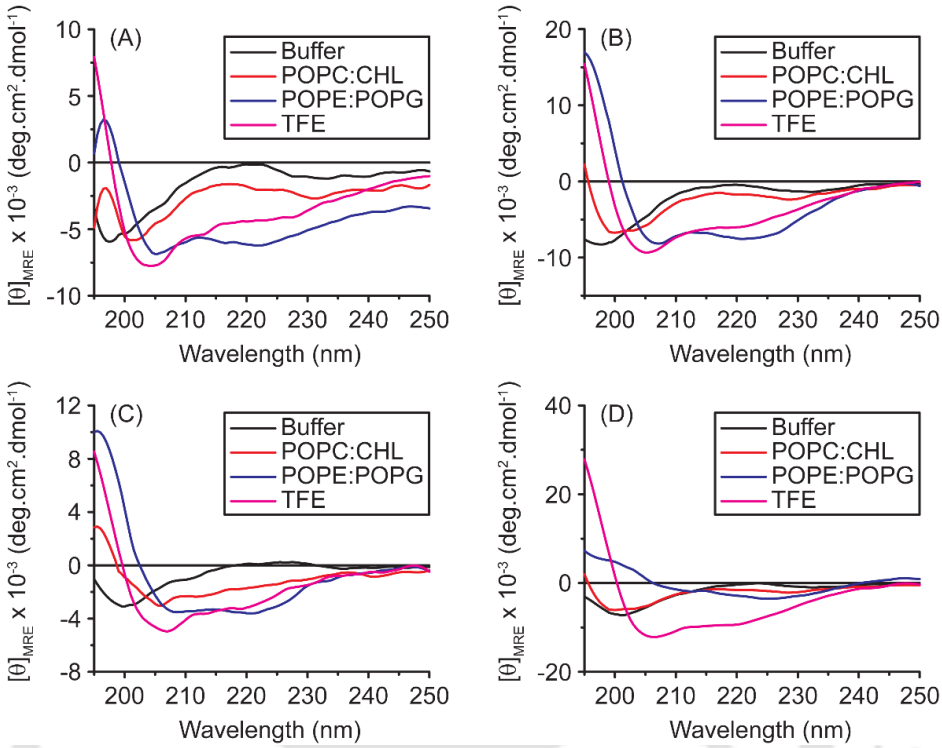

Figure 3.9 Circular dichroism spectra of the peptides. Spectra recorded in 10 mM phosphate buffer, pH 7.4 having 100 mM NaCl and 0.1 mM EDTA without and with lipid SUVs. Panels A, B, C, and D correspond to MreB$_{1-9}$, Ac-MreB$_{1-9}$, W-MreB$_{1-9}$, and Ac-W-MreB$_{1-9}$, respectively.

3.2.10 Hemolytic assay

Lysis of human erythrocytes by the peptides was examined at 50 and 100 μM peptide concentrations (Table 3.7). No hemolysis was caused by MreB$_{1-9}$, Ac-MreB$_{1-9}$, and W-MreB$_{1-9}$ at 50 μM concentration while ~4% hemolysis was caused by Ac-W-MreB$_{1-9}$. The lethal concentrations of W-MreB$_{1-9}$ and Ac-W-MreB$_{1-9}$ are ≤10 μM against all the organisms tested (Table 3.4). W-MreB$_{1-9}$ did not cause any hemolysis while Ac-W-MreB$_{1-9}$ caused ~4% hemolysis at 5-fold higher concentrations. W-MreB$_{1-9}$ caused little hemolysis (~1.6%) even at 10-fold higher concentration.

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Table 3.7. Percentage lysis of human erythrocytes by the MreB-derived peptide

Peptide concentration (μM)	Percentage hemolysis			
	$MreB_{1-9}$	Ac- $MreB_{1-9}$	W- $MreB_{1-9}$	Ac-W-$MreB_{1-9}$
50	0	0	0	4.15±0.96
100	0	0	1.62±0.08	11.88±2.44

3.2.11 FESEM analysis

Field emission scanning electron microscopic (FESEM) images of the peptide-treated microbes are shown in Figure 3.10. The peptide-treated microbes displayed unusual morphology. Treatment with peptides caused pore-like structures on the bacterial membrane alongside large-scale perturbation. Similar membrane perturbation was observed for *C. albicans* as well. The microscopic analysis suggests membrane perturbation as the mechanism of killing.

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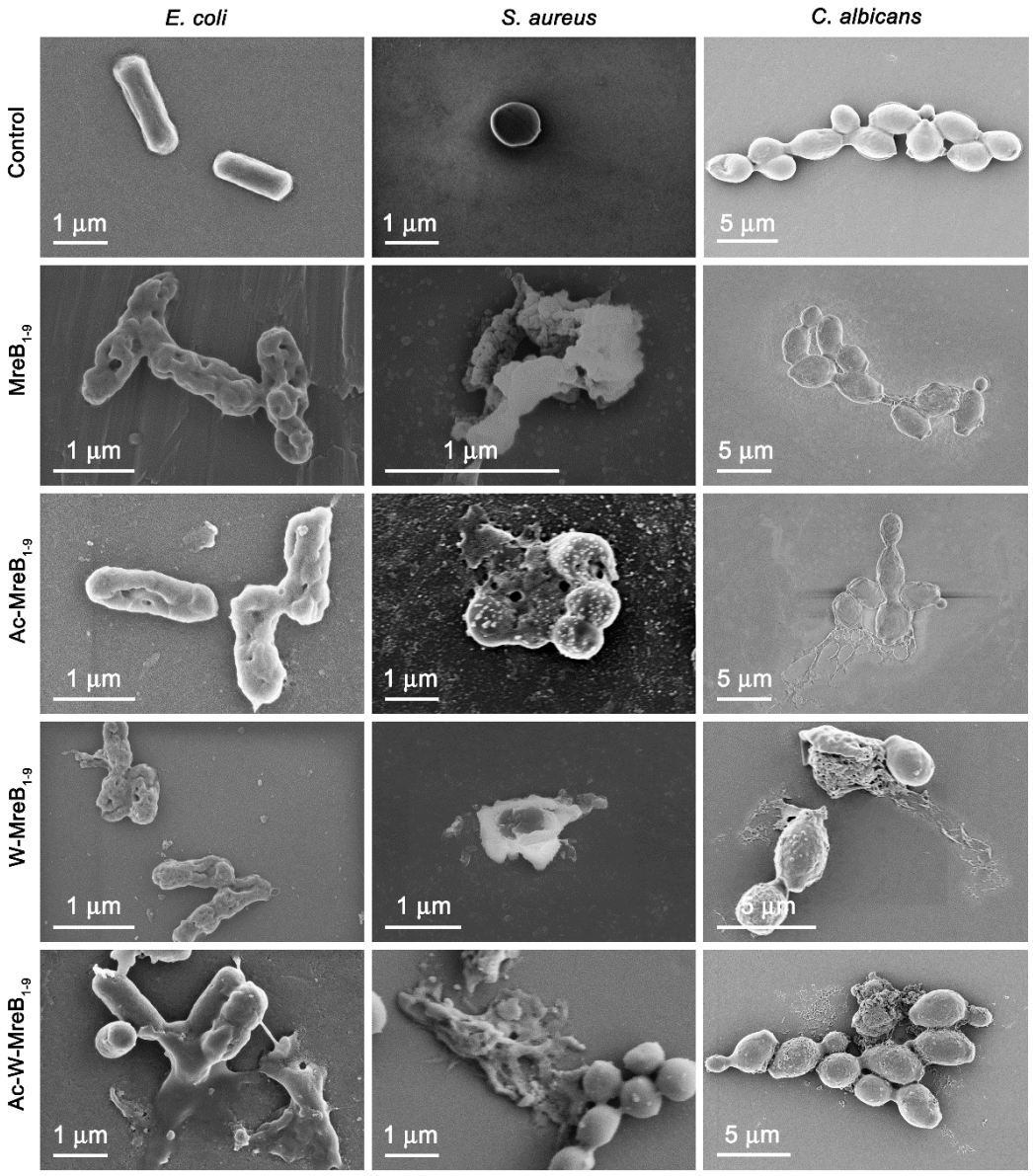

Figure 3.10 FESEM images of the peptide-treated *E. coli*, *S. aureus*, and *C. albicans*

3.2.12 Killing kinetics assay

Killing kinetics assay was performed with both bacteria and yeast. Peptides showed rapid killing in almost all the strains. All the peptides were highly active against *S. enterica* and instigated killing within 15 minutes of peptide treatment (Fig. 3.11). Peptide Ac-WMreB is proved to be the most effective one killing *E. coli*, *P. aeruginosa* in less than 5 minutes and *C. albicans* within 15 minutes of peptide treatment. However, in the case of *S. aureus*, more than 50 % of the bacteria survived till 1 hour of peptide treatment and got completely killed in 2 hours. Killing in *S. aureus* was slower compared to the other organisms tested.

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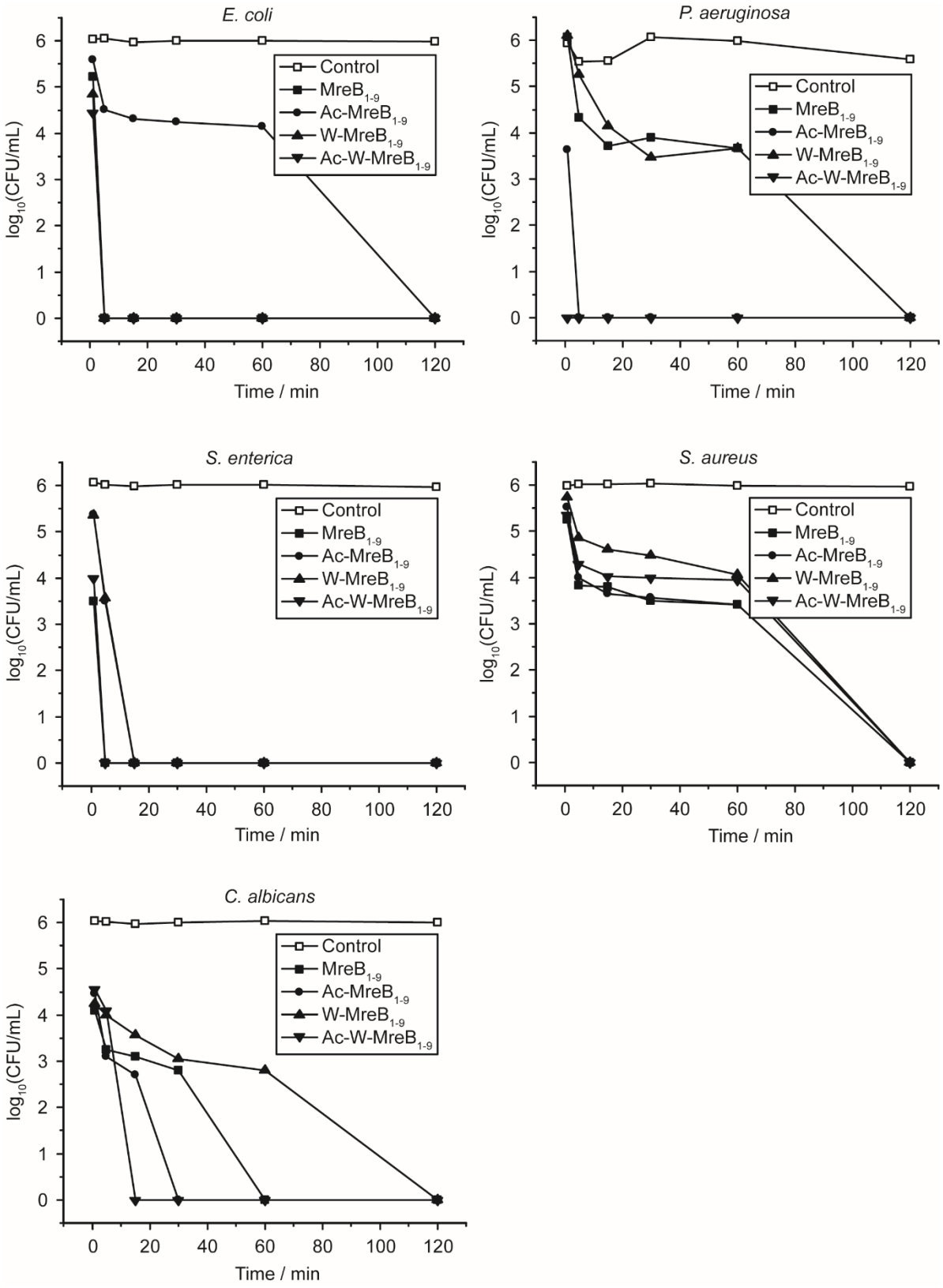

Figure 3.11 The kinetics of killing upon tratement with peptides at minimum lethal concentrations.

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3.3 Conclusion

In this chapter, I studied the antimicrobial and membrane-binding activity of the 9-residue long N-terminal amphipathic region of *E. coli* MreB protein and its analogs. The peptides displayed broad-spectrum activity, killing Gram-negative bacteria (*E. coli*, *P. aeruginosa*, *S. enterica*), Gram-positive bacteria (*S. aureus* and gentamicin-resistant MRSA), and fungus (*C. albicans*).

E. coli, *P. aeruginosa*, *S. enterica* are clinically-relevant Gram-negative bacteria. All the three bacteria were killed by the native sequence, $MreB_{1\text{-}9}$ at lethal concentrations comparable to many established antimicrobial peptides [216]. Extension of $MreB_{1\text{-}9}$ at the N-terminus by a tryptophan residue improved the activities drastically. All three bacteria could be completely killed at 5 μM or lower peptide concentration, an activity comparable to or better than many highly-active antimicrobial peptides [216]. Indolicidin, for example, displays an MIC of 67 μM against *P. aeruginosa*, *S. aureus*, and *C. albicans*; the MIC against *E. coli* was >134 μM. Human cathelicidin, LL-37 displays an MIC >50 μM against *E. coli*, *P. aeruginosa*, *S. aureus*, and *C. albicans*. Melittin, the highly lytic peptide from honey bee venom displays an MIC of 2.8 μM against *S. aureus*, 11.2 μM against *C. albicans*, and 22.4 μM against *E. coli* and *P. aeruginosa*. Cecropin A, an insect antimicrobial peptide, displays lethal concentrations of 0.32 μM against *E. coli* and 3.5 μM against *P. aeruginosa* [217]. PR-39, an AMP from pig intestine displays lethal concentrations of 0.3 μM against *E. coli* and 200 μM against *P. aeruginosa* and *S. aureus* [58].

The salt and divalent cations antagonised the activity of the peptides in a species-dependent manner. The outer membrane of Gram-negative bacteria contains lipopolysaccharide (LPS) as one of the major molecular components of the outer leaflet. The structure of LPS is characterized by a variable number of phosphate groups and anionic sugars in the oligosachharide core. The negatively charged groups are bridged by divalent cations [1, 218]. The antimicrobial peptides bind the LPS by displacing the divalent cations. The structural diversity of the LPS contribute to the differential susceptibility of the Gram-negative bacteria to the antimicrobial peptides.

Against Gram-positive bacterium, *S. aureus*, $MreB_{1\text{-}9}$ and its acetylated analog displayed lower activity compared to that against the Gram-negative bacteria. Gentamicin-resistant MRSA, however, could be efficiently killed at concentrations comparable to those required

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for killing Gram-negative bacteria. N-terminal extension by tryptophan drastically improves the activity wherein both methicillin-susceptible and resistant bacteria could be killed at ≤10 μM peptide concentrations. The most notable feature is that all the peptides retained >70% activity against *S. aureus* in the presence of salt and divalent cations. The peptides displayed excellent antifungal activity as well, killing *C. albicans* at concentrations as low as 5 μM. Candida is a clinically-important fungus, and *C. albicans* happens to be the most common species causing invasive candidiasis [219]. All the four peptides could efficiently kill *C. albicans* and retained substantial activity in the presence of salt and divalent cations. Lipid-binding assays, *E. coli* membrane-permeabilization assays, and FESEM imaging suggest membrane-permeabilization as the mechanism of killing.

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CHAPTER 4

Antimicrobial Peptides from C-terminal Amphipathic Region of *E. coli* FtsA Protein

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4.1 Summary

Antimicrobial properties of the peptides derived from N-terminal amphipathic helix of *E. coli* MreB protein were discussed in the previous chapter. The peptides turned out to be potent AMPs against Gram-negative and Gram-positive bacteria as well as opportunistic fungus, *C. albicans*. The results support the idea that membrane-interacting stretches of bacterial proteins can be engineered to combat the very same bacteria among others. Based on the results from one such peptide stretch, however, it may be primitive to conclude that such a concept would apply to all such peptides. In order to strengthen the proof of concept, I explored the antimicrobial potential of membrane-binding stretch from an unrelated bacterial protein, called FtsA, and the results are presented in this chapter. FtsA, an acronym for filamentous temperature sensitive A, is a protein present in most Gram-negative and Gram-positive bacteria. Structurally, FtsA resembles actin-like filaments and assists in cell division [220]. FtsA protein, through its conserved C-terminal amphipathic helix, helps in cytoplasmic membrane tethering of FtsZ polymers during cell division [221]. The absence of helix leads to the formation of long and stable FtsA polymer bundles in the cells that cannot take part in cell division [222]. I investigated the antimicrobial properties of the 10, 11, and 13-residue peptides derived from the amphipathic helical region of *E. coli* FtsA protein against *E. coli*, *S. enterica*, *P. aeruginosa*, gentamicin-resistant *S. aureus* (gentamicin-resistant MRSA), and *C. albicans*. The peptides display preferential binding to the negatively charged lipid vesicles and kill all the organism tested. Membrane permeabilization is suggested as one of the mechanisms of antimicrobial action.

4.2 Results

4.2.1 Peptides

The amino acid sequences and IDs of the 10 ($FtsA_{409-418}$), 11 ($FtsA_{408-418}$), and 13-residue ($FtsA_{406-418}$) peptides derived from *E. coli* FtsA protein are shown in Table 4.1. Helical wheel-projections of the 13-residue peptide is shown in Figure 4.1.

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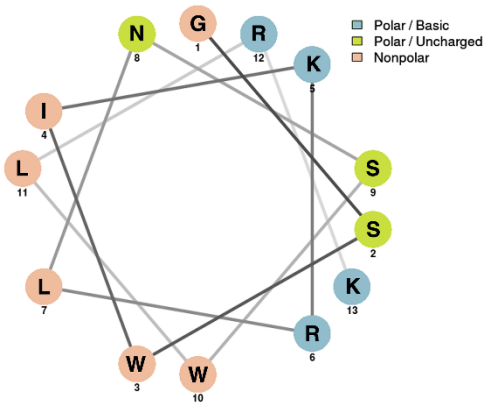

Figure 4.1 Helical wheel representation of FtsA13 peptide derived from *E. coli*

Mean hydrophobicity (<H>) and mean hydrophobic helical moments (<μH>) were calculated using Heliquest web server [215]. The discrimination factor, D was calculated using formula, D = 0.944 <μH> + 0.33(z). As the discrimination factor is larger than 1.34, all the peptides were predicted to be membrane-binding ones.

Table 4.1. Sequences and physicochemical properties of the peptides

Peptide	Peptide sequence[a]	z[b]	<H>	<μH>	D
FtsA10	IKRLNSWLRK-am	+5	0.281	0.761	2.37
Ac-FtsA10	Ac-IKRLNSWLRK-am	+4	0.281	0.761	2.04
FtsA11	WIKRLNSWLRK-am	+5	0.460	0.843	2.45
Ac-FtsA11	Ac-WIKRLNSWLRK-am	+4	0.460	0.843	2.12
FtsA13	GSWIKRLNSWLRK-am	+5	0.386	0.716	2.33
Ac-FtsA13	Ac-GSWIKRLNSWLRK-am	+4	0.386	0.716	2.00

[a] 'Ac-' at N-terminus indicates amino-terminal acetylation, while '-am' at C-terminus indicates C-terminal amide.
[b] Net charge on the peptide at pH 7.4

The peptides were synthesized, purified using reversed-phase HPLC (Figure 4.2), and their identities ascertained using MALDI-TOF mass spectrometry (Figure 4.3).

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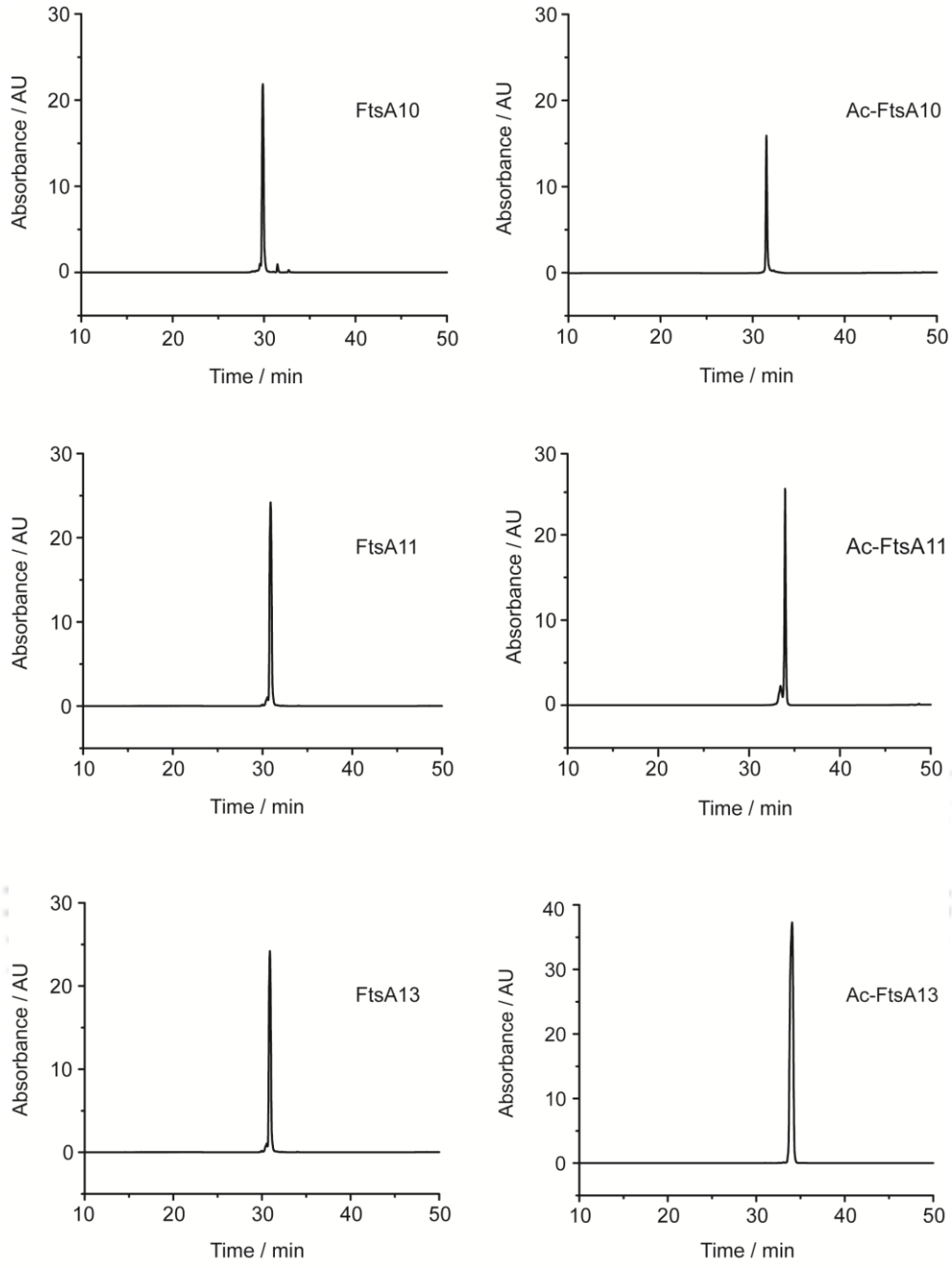

Figure 4.2 Reversed-phase HPLC chromatograms of the purified FtsA-derived peptides.

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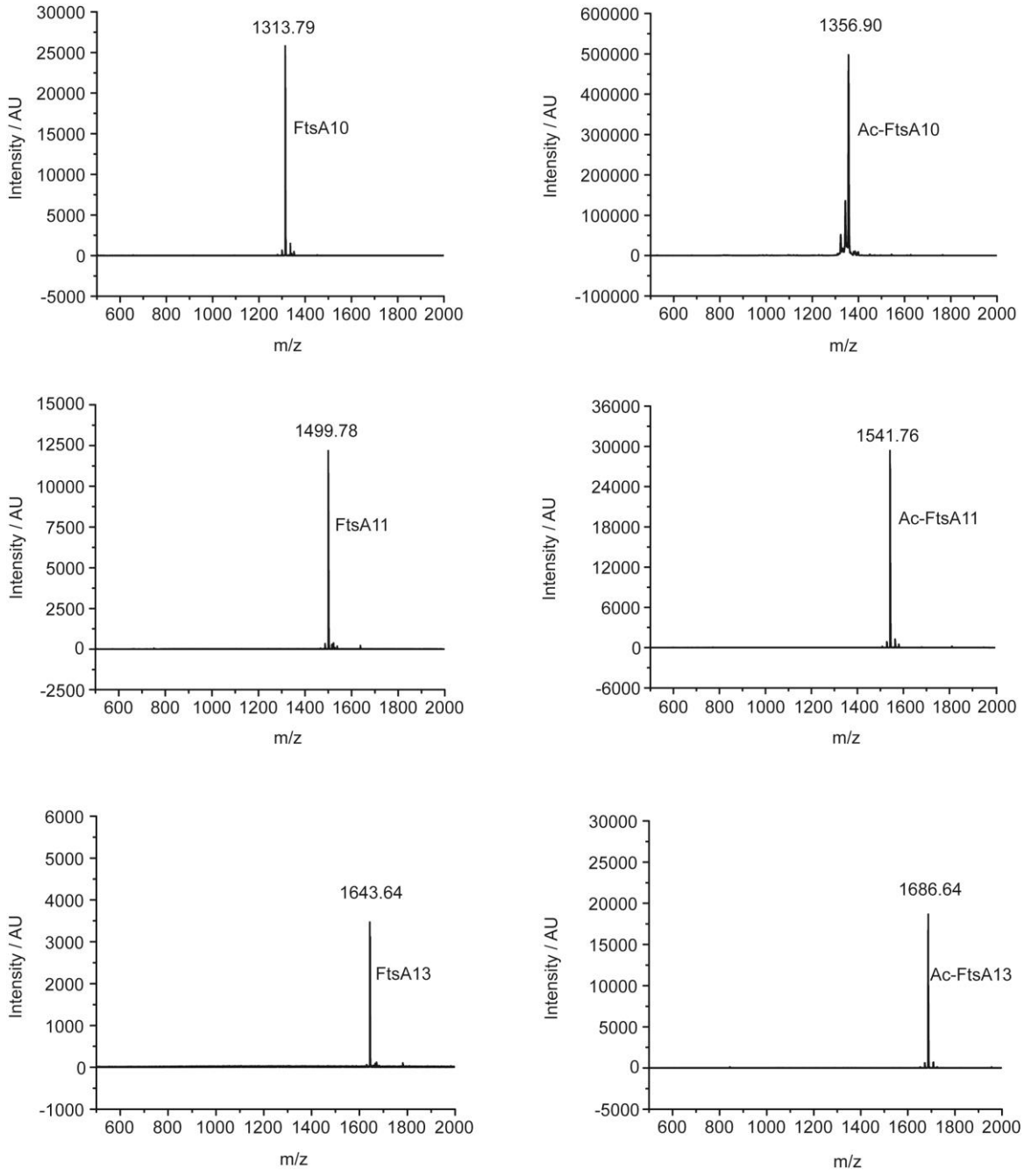

Figure 4.3 MALDI-TOF mass spectra of the purified FtsA-derived peptides

4.2.2 Tryptophan fluorescence

Binding of peptides to lipid vesicles was examined using steady-state tryptophan fluorescence. Fluorescence emission spectra were recorded in HEPES-buffered saline (HBS) in the absence of lipid vesicles and in the presence of POPC:CHL (10:1) and POPE:POPG (7:3) SUVs at peptide to lipid ratios of 1:50 and 1:100 (Figure 4.4).

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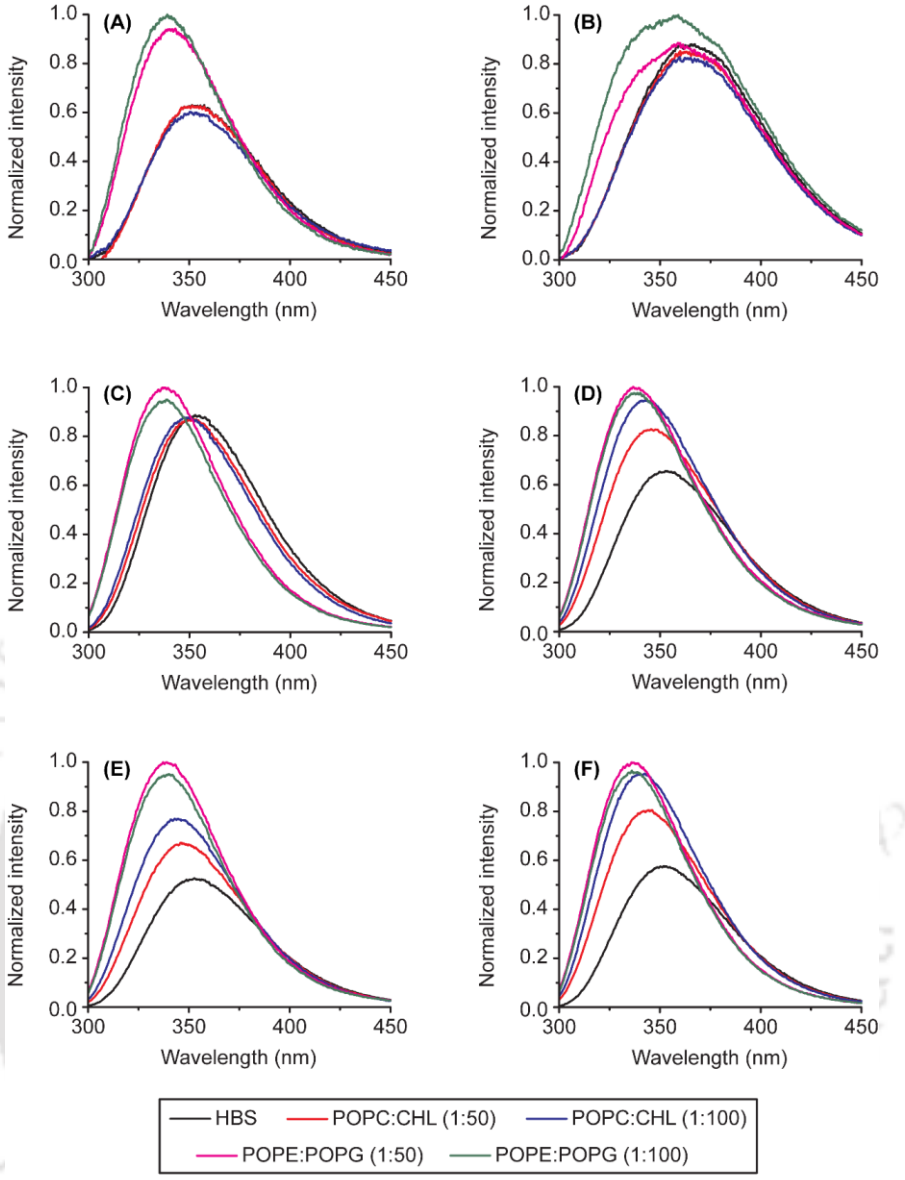

Figure 4.4 Tryptophan fluorescence emission spectra of FtsA-derived peptides. (A) FtsA10, (B) Ac-FtsA10, (C) FtsA11, (D) Ac-FtsA11, (E) FtsA13, and (F) Ac-FtsA13.

In HBS, all the peptides except Ac-FtsA10 display emission maximum ~353 nm; Ac-FtsA10 displays emission maximum ~363 nm. In the presence of POPC:CHL SUVs, FtsA10 and Ac-FtsA10 display no appreciable change in the emission spectra. In the presence of POPE:POPG vesicles, however, distinct blue shift with enhancement in fluorescence intensity is observed suggesting binding to the lipid vesicles. FtsA11 shows small (≤5 nm) blue shift in the presence of POPC:CHL vesicles but a large blue shift (~15 nm) is observed with POPE:POPG vesicles. The data indicate preferential binding to the negatively charged

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vesicles. Ac-FtsA11, FtsA13, and Ac-FtsA13 also display preferential binding to negatively charged SUVs; there is, however, appreciable binding to POPC:CHL SUVs as well.

4.2.3 Tryptophan quenching

Accessibility of tryptophan side-chains to solvent was assessed using dynamic quencher acrylamide. The Stern-Volmer plots for all the peptides were fit linearly (Figure 4.5).

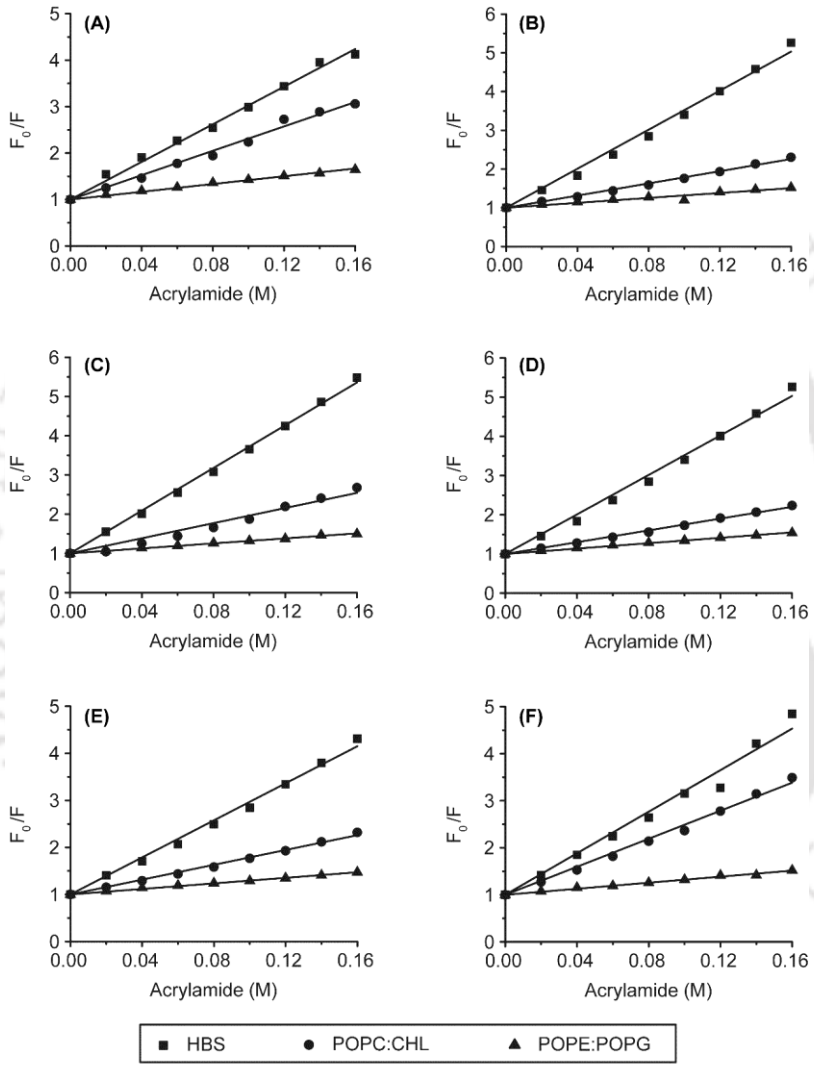

Figure 4.5 Stern Volmer plot for tryptophan quenching by acrylamide. Where, (A) FtsA10, (B) Ac-FtsA10, (C) FtsA11, (D) Ac-FtsA11, (E) FtsA13, and (F) Ac-FtsA13.

K_{SV} values of peptides decrease in the presence of lipid vesicles suggesting membrane binding. Tryptophan is better shielded from quenching in the presence of negatively charged vesicles. Net accessibility factors (NAF) were determined using the formula: NAF = K_{SV} (with SUVs)/K_{SV} (without SUVs) and are shown in Table 4.2. The extent of quenching is lesser in POPE:POPG vesicles compared to that in POPC:CHL vesicles.

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Table 4.2 K_{sv} and NAF values for all the FtsA-derived peptides

Peptides	HBS		POPC:CHL		POPE:POPG	
	K_{sv} (M^{-1})	NAF	K_{sv} (M^{-1})	NAF	K_{sv} (M^{-1})	NAF
FtsA10	20.24	1	13.11	0.64	4.17	0.20
Ac-FtsA10	25.19	1	7.86	0.31	3.17	0.12
FtsA11	27.24	1	9.64	0.35	3.19	0.11
Ac-FtsA11	25.19	1	7.52	0.29	3.44	0.13
FtsA13	19.68	1	7.88	0.40	2.94	0.14
Ac-FtsA13	22.07	1	14.89	0.67	3.21	0.14

4.2.4 Antimicrobial assay

Antimicrobial activity of peptides was tested against *E. coli*, *S. enterica*, and *P. aeruginosa* (Gram-negative bacteria); gentamicin-resistant MRSA (Gram-positive bacterium), and *C. albicans* (an opportunistic yeast). MIC values against these microbes are shown in Table 4.3. The peptides, by and large, display better activity against Gram-negative bacteria followed by *C. albicans* and gentamicin-resistant MRSA. FtsA10 displays an MIC of 64 μM against *E. coli* and *C. albicans*. N-terminal acetylation of the peptide improves the activity against *E. coli* (32 μM), *S. enterica* (16 μM) and *P. aeruginosa* (32 μM). Both the 10-residue peptides, however, fail to inhibit the growth of gentamicin-resistant MRSA even at 128 μM concentration.

Table 4.3 MIC of the FtsA-derived peptides

Peptides	Minimum inhibitory concentration (μM)				
	E. coli	*S. enterica*	*P. aeruginosa*	Gentamicin-resistant MRSA	*C. albicans*
FtsA10	64	128	128	>128	64
Ac-FtsA10	32	16	32	>128	64
FtsA11	8	8	16	64	16
Ac-FtsA11	2	8	8	32	8
FtsA13	2	4	4	32	4
Ac-FtsA13	4	4	4	32	8

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The 11 and 13-residue peptides, on the other hand, exhibited activity against all five microorganisms. All the four peptides displayed strong activity against *E. coli and S. enterica* with MIC values ≤8 μM and against *P. aeruginosa* and *C. albicans* with MIC values ≤16 μM. The activity against gentamicin-resistant MRSA, however, was much lower; FtsA11 displayed an MIC of 64 μM whereas the other three peptides displayed MIC of 32 μM.

4.2.5 Salt sensitivity assay

The sensitivity of peptides to salt and divalent cations was assessed by determining the MIC values in the presence of 100 mM NaCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$ as described in section 2.12.2, chapter 2. The data are shown in Table 4.4. The activity of FtsA10 and Ac-FtsA10 got reduced in the presence of salt and divalent cations. However, it is interesting to note that the MIC values of 11 and 13-residue peptides against *E. coli* are ≤8 μM under these conditions as well. Ac-FtsA11 has retained its activity against *S. enterica* in the presence of both salt and divalent cations (8 μM). In general, the MIC of the 11 and 13-residue peptides against *S. enterica* and *P. aeruginosa* are ≤16 μM and ≤32 μM. MIC of Ac-FtsA11 and FtsA13 against gentamicin-resistant MRSA is same in the presence of salt and divalent cations (32 μM). Against *C. albicans*, FtsA13 turns out to be the most potent peptide; the peptide displays an MIC of 16 μM in the presence of $CaCl_2$ while lower MICs were obtained in the presence of NaCl and $MgCl_2$.

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Table 4.4 MIC (μM) of the FtsA-derived peptides in the presence of salt and divalent cations.

Microbe → Peptide ↓		*E. coli*	*S. enterica*	*P. aeruginosa*	*Gentamicin-resistant MRSA*	*C. albicans*
FtsA10	NaCl	128	128	>128	>128	>128
	$MgCl_2$	64	128	128	>128	128
	$CaCl_2$	128	>128	>128	>128	>128
Ac-FtsA10	NaCl	64	32	64	>128	128
	$MgCl_2$	32	32	64	>128	64
	$CaCl_2$	64	32	64	>128	>128
FtsA11	NaCl	8	16	16	128	32
	$MgCl_2$	8	8	16	128	16
	$CaCl_2$	8	8	32	128	64
Ac-FtsA11	NaCl	8	8	16	32	16
	$MgCl_2$	4	8	8	32	16
	$CaCl_2$	4	8	16	32	32
FtsA13	NaCl	8	4	8	32	8
	$MgCl_2$	2	4	8	32	4
	$CaCl_2$	4	8	16	32	16
Ac-FtsA13	NaCl	8	8	16	32	8
	$MgCl_2$	8	8	8	64	8
	$CaCl_2$	8	8	16	64	32

4.2.6 Outer membrane permeabilization assay

Permeabilization of *E. coli* outer membrane was assayed using NPN fluorometric assay. Polymyxin B (10 μg/ml) was used as positive control and NPN uptake caused by polymyxin B was taken as 100% uptake. Table 4.5 shows the percentage NPN uptake by *E. coli* cells at different peptide concentrations.

Table 4.5 Outer membrane permeabilization of *E. coli* cells by FtsA-derived peptides. The values in parentheses indicate the MIC of peptides against *E. coli*.

Peptide conc. (μM)	FtsA10 (64 μM)	Ac-FtsA10 (32 μM)	FtsA11 (8 μM)	Ac-FtsA11 (2 μM)	FtsA13 (2 μM)	Ac-FtsA13 (4 μM)
2	49.76 ± 22.62	87.3 ± 7.84	63.5 ± 6.9	82.95 ± 18.99	100	43.76 ± 12.33
4	100	100	68.8 ± 12.4	85.56 ± 14	100	50.84 ± 17.85
8	—	—	71.2 ± 15.06	98.40 ± 2.25	—	97.79 ± 3.12

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All the peptides exhibit >40% uptake at 2 μM concentration; FtsA13 displayed 100% uptake. FtsA10 and Ac-FtsA10 resulted in 100% uptake at 4 μM concentration. The other three peptides did not result in 100% uptake up to 8 μM concentration; Ac-FtsA11 and Ac-FtsA13, however, displayed ~98% uptake. FtsA11 caused similar NPN uptake (~60-70%) at all the three concentrations tested. It is interesting to note that FtsA10 and Ac-FtsA10 display 100% uptake at 4 μM concentration whereas they display MIC values of 64 μM and 32 μM respectively. Contrarily, FtsA11, Ac-FtsA11, Ac-FtsA13 display ~71%, 83%, and 51% NPN uptake at their MIC. The data indicate that outer membrane permeabilization alone cannot be correlated to the antimicrobial activity.

4.2.7 Inner membrane permeabilization assay

The voltage sensitive dye $DiSC_3(5)$ was used to determine the depolarization of *E. coli* inner membrane. Polymyxin B (10 μg/ml) served as the positive control in the assay. Fluorescence emission intensity from $diSC_3(5)$-treated *E. coli* cells was recorded at 670 nm after exciting the samples at 622 nm (slit width = 1 nm). All the peptides other than FtsA10 caused gradual increase in $diSC_3(5)$ fluorescence intensity indicating inner membrane permeabilization (Figure 4.6). Despite being an efficient outer membrane-permeabilizing agent, FtsA10 failed to cause a noticeable increase in $diSC_3(5)$ fluorescence. The differential behavior could possibly arise due to peptide's binding strength to the outer membrane. The peptide possesses a high charge density compared to the other peptides, and the charge is concentrated near the peptide termini, which could influence the way the peptide interacts with the outer membrane. Strong binding to outer membrane lipids could limit the peptide's access to inner membrane. Ac-FtsA10 shows a gradual increase in the fluorescence intensity indicating permeabilization of the inner membrane (panel B). Similar enhancement is observed for FtsA11 (panel C), Ac-FtsA11 (panel D), FtsA13 (panel E), and Ac-FtsA13 (panel F). Polymyxin B causes similar enhancement in $diSC_3(5)$ fluorescence (panel G) indicating that the peptides are as effective membrane-permeabilizing agents as polymyxin B. Bacterial cells do not show any change in the $diSC_3(5)$ fluorescence on the experimental time-scale (negative control, panel H). These data infer membrane permeablization by the peptides as the mechanism of antimicrobial action.

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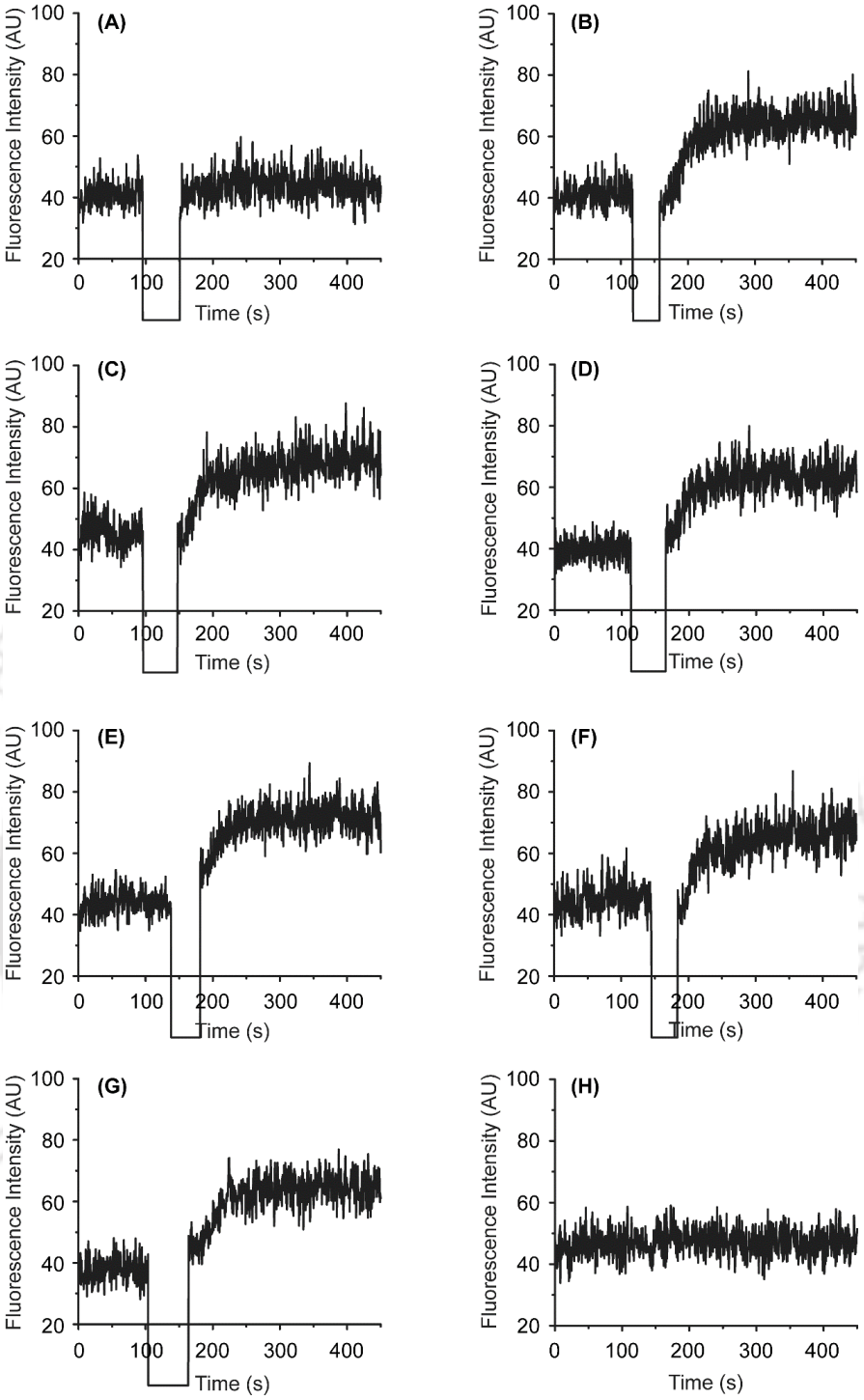

Figure 4.6 Inner membrane permeabilization of *E. coli* cells by FtsA-derived peptides. (A) FtsA10, (B) Ac-FtsA10, (C) FtsA11, (D) Ac-FtsA11, (E) FtsA13, (F) Ac-FtsA13, (G) Polymyxin B and (H) Cells without peptide.

4.2.8 Circular dichroism:

CD spectroscopic analysis shows that the peptides are unordered in 10 mM phosphate buffer, pH 7.4 having 100 mM NaCl and 0.1 mM EDTA (Figure 4.7). In the presence of

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POPC:CHL SUVs, all the peptides are largely unordered. Ac-FtsA11 and Ac-FtsA13, however, appear to become somewhat structured with a shift of the ~200-202 nm band to longer wavelength and higher negative ellipticity around 222 nm. In the presence of POPE:POPG SUVs, all the peptides except FtsA10 become structured. Ac-FtsA10 shows a positive ~195 nm and negative bands around 207 and 223 nm indicating a distinct α-helical conformation (panel B). The 11 and 13-residue peptides display CD spectra with a broad negative band centered between 225-230 nm with a shoulder around 210 nm (panels C-F). The positive band appears between 197-200 nm. Such spectra have been assigned to β-turns in literature [223, 224]. Even though a β-turn can be mediated by the Asn-Ser motif present in the peptide, the resulting β-hairpin structure would be destabilized by the electrostatic repulsion between lysine residue near N-terminus and the Arg residue near C-terminus. We, therefore don't interpret these spectra as the ones arising from β-turns. Such spectrum has also been reported for the major coat protein of the filamentous phage fd. The fd protein is an all helical protein but displays CD spectrum with an intense band around 222 nm with a shoulder around 208 nm [225]. Oxidation of Trp with N-bromosuccinimide resulted in the classical α-helical spectrum. Trp was found to contribute a strong negative band around 222 nm and a positive band around 210 nm thereby distorting the spectrum. An ideal α-helical conformation for the 11 and 13-residue FtsA peptides would place the two tryptophan residues in closed proximity (Cα distance ~10.5 Å). The coupled-oscillator interaction in the clusters of aromatic residues has been reported to significantly contribute to far-UV CD spectra of proteins and peptides. We, therefore, assign these spectra to the α-helical conformation.

In TFE, Ac-FtsA10, Ac-FtsA11, FtsA13 and Ac-FtsA13 show distinct α-helical conformation. FtsA10 becomes somewhat structured in TFE (panel A). Interestingly, FtsA11 does not show the spectrum characteristic of an α-helical conformation; a minimum around 205 nm and a broad shoulder ranging from 210-222 nm indicates a mixture of α-helical and unordered conformation of the peptide in TFE (panel C).

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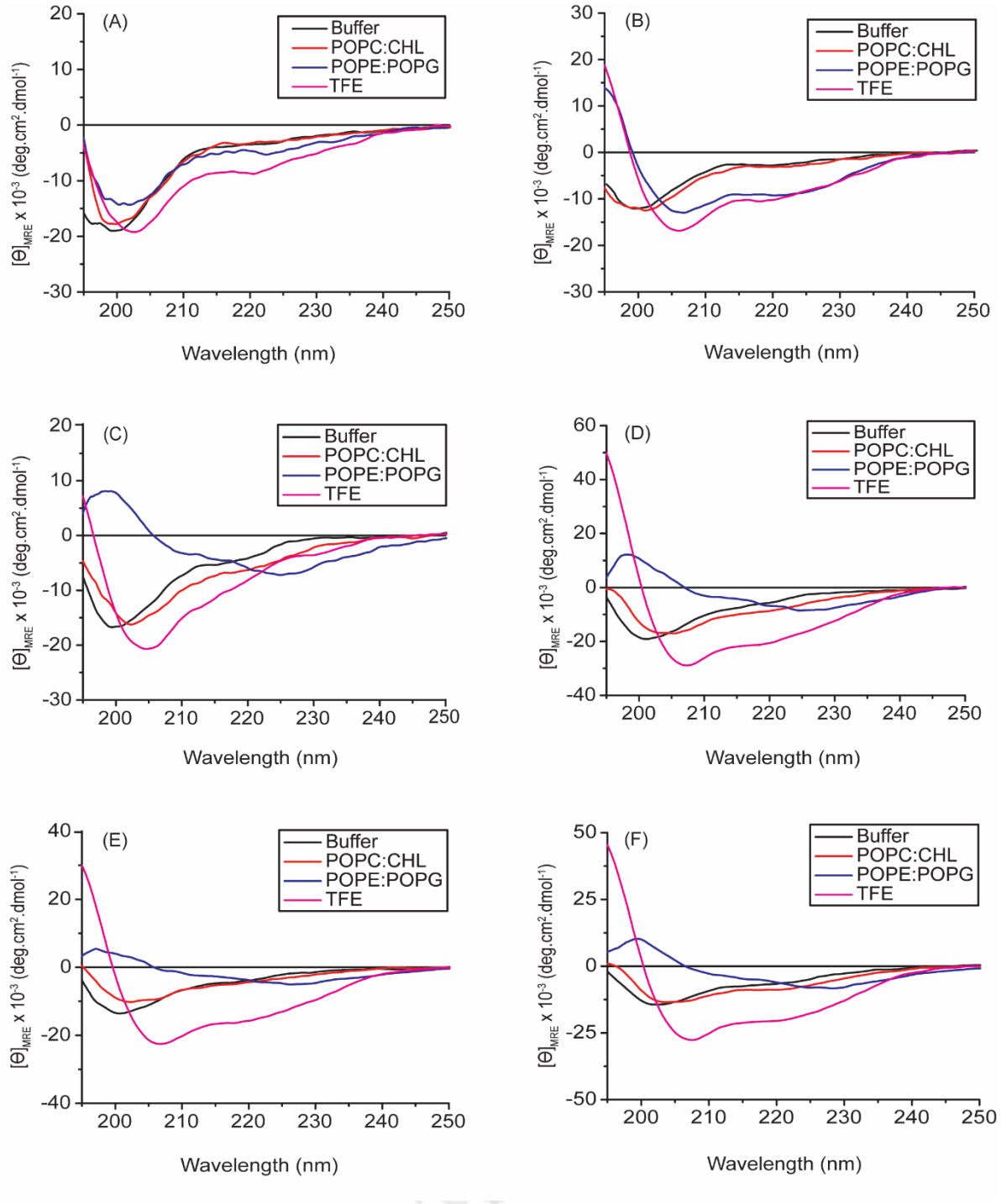

Figure 4.7 Circular dichroism spectra of the peptides. Spectra recorded in 10 mM phosphate buffer, pH 7.4 having 100 mM NaCl and 0.1 mM EDTA without and with lipid SUVs. Panels A, B, C, D, E, and F corresponds to FtsA10, Ac-FtsA10, FtsA11, Ac-FtsA11, FtsA13, Ac- FtsA13 respectively.

4.2.9 Hemolytic assay

Toxicity of peptides was tested against human erythrocytes as described in section 2.13, chapter 2. None of the peptides caused more than 1.5% hemolysis up to 32 μM

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concentration (Table 4.6). These data indicate that 11 and 13-residue peptides are promising antimicrobial candidates.

Table 4.6 Percentage lysis of human erythrocytes by the FtsA-derived peptides

Peptide conc. (μM)	Percentage hemolysis					
	FtsA10	Ac-FtsA10	FtsA11	Ac-FtsA11	FtsA13	Ac-FtsA13
2	0	0	0	0	0.23±0.21	0
8	0.2±0.04	0.09±0.02	0	0.17±0.06	0.42±0.05	0
32	0.22±0.09	0.15±0.08	0.31±0.21	0.32±0.01	1.18±0.77	0.63±0.43

4.2.10 FESEM analysis

FESEM imaging of the peptide-treated microbes shows abnormal cell morphology compared to the untreated microbes (Figure 4.8). The data suggest membrane-permeabilization as the possible mechanism of peptide action that is in correlation with *E. coli* membrane permeabilization assays.

4.3 Conclusion

The peptides derived from C-terminal amphipathic helix of *E. coli* FtsA protein were predicted to be membrane-binding ones by Heliquest analysis [215]. The peptides exhibit preferential binding to the negatively charged lipid vesicles as indicated by tryptophan fluorescence studies. The 10-residue peptide, FtsA10 displays MIC ≥64 μM against all the organisms tested indicating poor antimicrobial potential. N-terminal acetylation causes little improvement in the activity. The 11 and 13-residue peptides, on the other hand, turned out to be potent antimicrobials. All the four peptides show MIC values ≤8 μM against *E. coli* and *S. enterica*. *P. aeruginosa* was also killed by Ac-FtsA11, FtsA13, and Ac-FtsA13 at 8 μM concentration; FtsA11, on the other hand, displayed a MIC of 16 μM against *P. aeruginosa*. MIC values against gentamicin-resistant MRSA turned out to be at least 4-fold higher indicating selectivity towards Gram-negative bacteria. This is quite intriguing as Gram-positive bacteria, in general, have a higher content of negatively charged lipids and most AMPs reported in the literature show better activity against Gram-positive bacteria [139].

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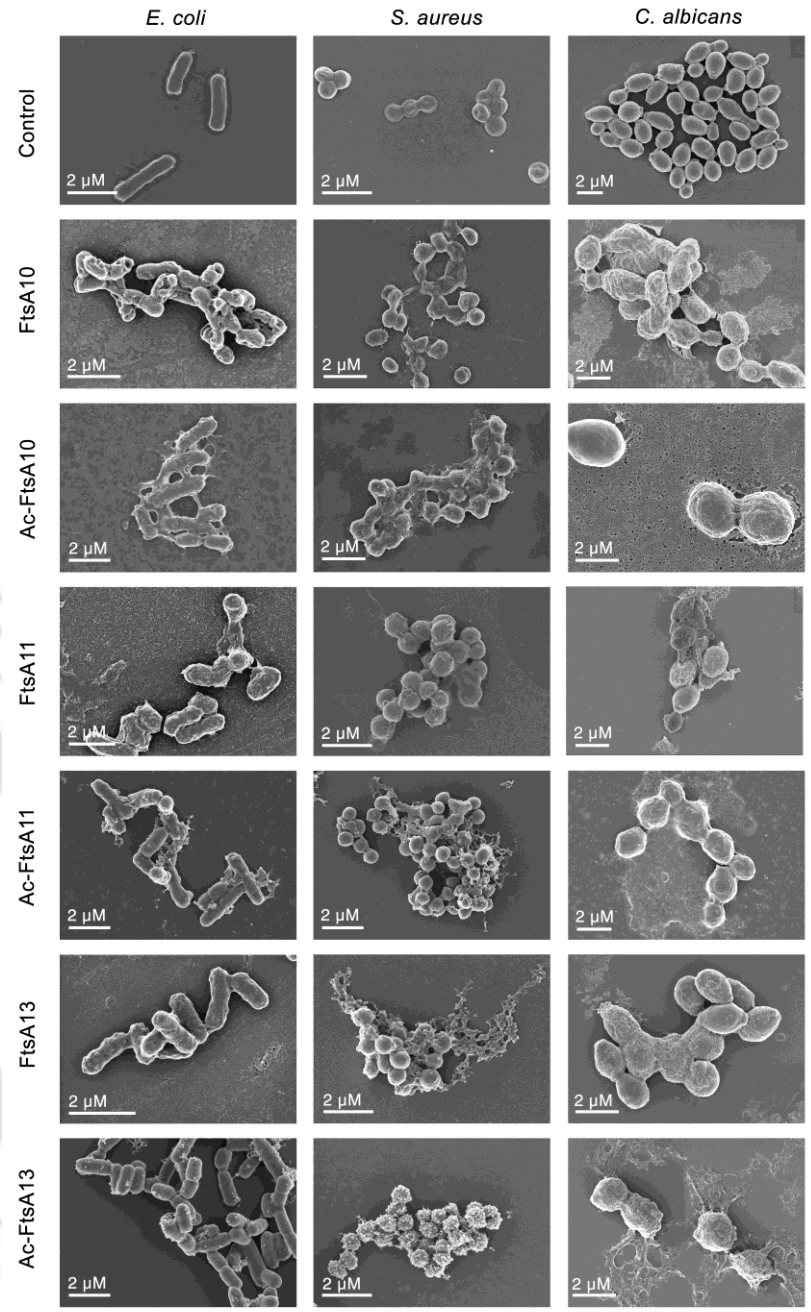

Figure 4.8 FESEM images of the peptide-treated microbes

We used gentamicin-resistance MRSA in this study as the model Gram-positive bacterium. *S. aureus* membrane, however, can have very high content of cationic lipid, lysyl-phosphatidylglycerol thereby diminishing the overall charge of the membrane thereby countering the AMPs. Against MreB-derived peptides, however, the very same bacteria displayed susceptibility very similar to that displayed by Gram-negative bacteria. These data hint that the physicochemical properties of peptides could impart this selectivity. The *E. coli* FtsA-derived peptides used in this study are highly amphipathic but only

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moderately hydrophobic; less hydrophobic peptides could display selectivity towards Gram-negative bacteria [139, 226]. Furthermore, it is likely that membrane permeabilization is not the sole mechanism of killing, the peptides may interfere with the intracellular molecules once they permeabilize the cytoplasmic membrane of the bacteria. Other than bacteria, the 11- and 13-residue AMPs could inhibit the opportunistic fungus, *C. albicans* as well at promising MIC ≤16 μM. The data obtained with MreB and FtsA-derived peptides support the hypothesis that membrane-binding stretches of microbial proteins could turn out to be novel membrane-permeabilizing antimicrobial agents. Such peptides can kill the very same microbe by permeabilizing the membrane and possibly by disturbing the homeostasis if they bind to the intracellular targets of the parent protein. In addition to that, the peptides could combat other microbes as well through usual membrane permeabilization mechanism as observed for both MreB and FtsA-derived peptides.

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CHAPTER 5

Aromatic Residue-Rich Antimicrobial Peptide Derived from C-terminal Region of *B. subtilis* LCI Peptide

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5.1 Summary

Bacteria are a great source of AMPs. Bacterial AMPs, referred to as bacteriocins, are produced by a large number of bacteria to protect themselves from invading pathogens. Bacteriocins exhibit antimicrobial activity at very low concentrations making them promising candidates for next-generation antibiotics [227].

LCI is an AMP produced by *Bacillus subtilis* strain A014 [63]. The peptide inhibits plant pathogens, *Xanthomonas* and *Pseudomonas*. The peptide harbors certain interesting structural features *viz.* an unusually high thermodynamic stability and richness in aromatic residues. The peptide folds into a highly stable structure characterized by a four-stranded β-sheet [64]. Beta-sheet rich AMPs are usually stabilized by one or more disulfide linkages. LCI, however, lacks cysteine but is reported to have retained >80% activity even after 20 minutes of heating at 80 ºC. Such remarkable stability for a peptide lacking a disulfide linkage is intriguing. LCI is unusually rich in aromatic residues; 10 out of 47 *i.e.* 21% of the residues are aromatic. The high thermodynamic stability of LCI is believed to be conferred by aromatic stacking interactions, cation-π interactions, and aromatic-backbone amide interactions. The distribution of cationic and aromatic residues along the sequence is worth noticing; the N-terminal 21-residue stretch harbors only 2 Phe and 1 Lys residues. The C-terminal 26 residue stretch, on the other hand, harbors 3 Trp, 4 Tyr, 1 Phe, 5 Lys, and 1 Arg residues. This implies that ~86% of the cationic residues and 80% of the aromatic residues lie in the C-terminal 26-residue stretch. This stretch contributes two β-stands, β3 and β4 to the native LCI structure wherein the two strands are linked through a type-I β-turn. Such peculiar properties prompted us to investigate the antibacterial properties of C-terminal 26-residue stretch. We synthesized the C-terminal amidated peptide (KWIFKSKYYDSSKGYWVGIYEVWDRK-NH_2), referred to as LCI_{22-47} hereafter, and investigated the antibacterial activity against *E. coli*, gentamicin-resistant MRSA, and plant pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). The peptide killed all the three organisms efficiently with lethal concentrations of 4 μM or less. Studies carried out with model membranes and *E. coli* membranes suggest membrane-permeabilization as one of the mechanisms of killing.

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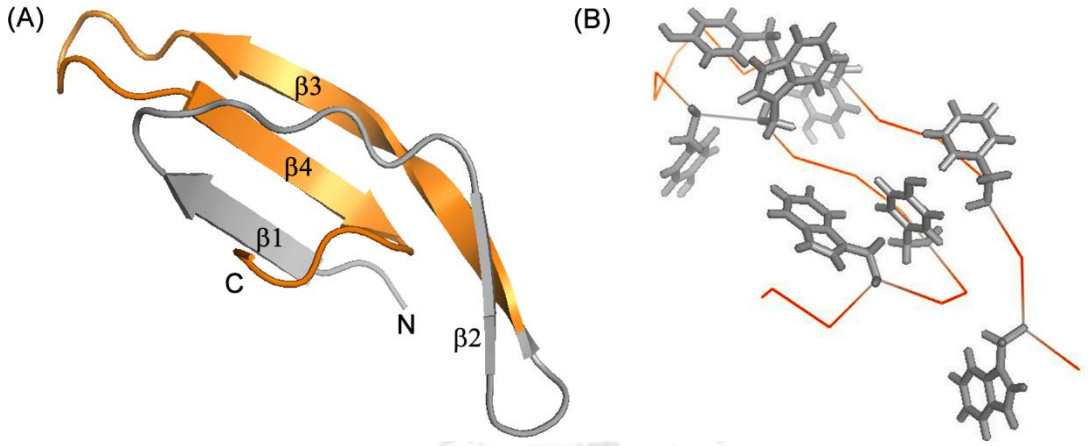

Figure 5.1 Structure of *Bacillus subtilis* LCI. Panel A shows the NMR structure of full-length LCI (PDB ID: 2B9K). Panel B shows the 22-47 residue region to highlight the distribution of aromatic residues. This is not to imply that the LCI_{22-47} retains the same structure as a fragment.

5.2 Results

5.2.1 Peptide

LCI_{22-47} was synthesized, purified using reversed-phase HPLC, and molecular mass determined using MALDI-TOF mass spectrometry. The HPLC chromatogram and the MALDI-TOF mass spectrum of the purified peptide are shown in figure 5.2.

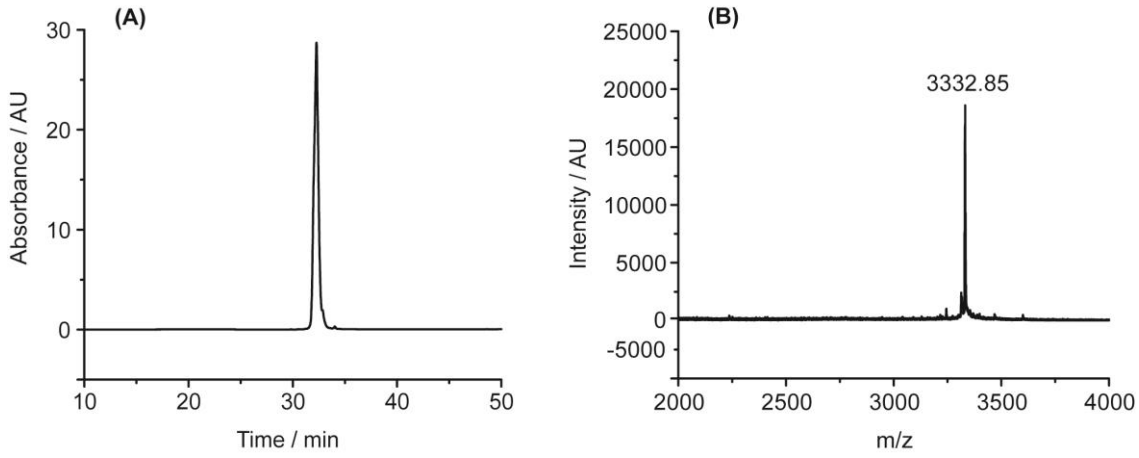

Figure 5.2: Reversed-phase HPLC chromatogram (panel A) and MALDI-TOF mass spectrum of LCI_{22-47} (panel B).

5.2.2 Antibacterial activity

As the peptide gets precipitated in Muller Hinton broth, the antibacterial assay was carried out in using colony count method as described in section 2.10.1, chapter 2. The activity

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against *E. coli* and gentamicin-resistant MRSA was assayed in nutrient medium. *Xoo*, on the other hand, was grown in peptone-sucrose growth medium having 0.1 wt% sodium glutamate and the assay was carried out in agar plates prepared with the same medium. The activity was determined against gentamicin-resistant MRSA as the model organism for Gram-positive bacterium and *E. coli* and *Xoo* as the model organisms for Gram-negative bacteria. The peptide displayed potent activity against all the three bacteria; the complete killing of *E. coli* was achieved at 2 μM peptide concentration whereas gentamicin-resistant MRSA and *Xoo* were killed at a concentration of 4 μM.

5.2.3 Salt sensitivity assay

The activity of peptide in the presence of salt and divalent cations was carried out as described in section 2.12.1, chapter 2. Antibacterial activity gets compromised in the presence of salt and divalent cations (Table 5.1). In 1 mM $MgCl_2$, the peptide retains >80% activity at its lethal concentration against all the organisms tested. In 100 mM NaCl, however, the activity is reduced to ~41% and ~58% against *E. coli* and *Xoo*, respectively. The activity against gentamicin-resistant MRSA, however, gets completely lost at its lethal concentration. In 2 mM $CaCl_2$, the peptide's activity is reduced to ~50% against *E. coli* and gentamicin-resistant MRSA but completely lost against *Xoo*. Complete loss of activity in the presence of salt or divalent cations does not imply that the peptide cannot kill the microbe; it usually implies that the microbe will be killed at a higher concentration. To confirm this, the activity of peptide against gentamicin-resistant MRSA in 100 mM NaCl and against *Xoo* in 2 mM $CaCl_2$ was determined at twice the minimum lethal concentration *i.e.* at 8 μM concentration. At 8 μM peptide concentration, the peptide killed ~90% gentamicin-resistant MRSA in 100 mM NaCl and ~45% *Xoo* in 2 mM $CaCl_2$.

Table 5.1 Percentage killing of bacteria in the presence of salt and divalent cations

	E. coli	**Gentamicin-resistant MRSA**	***Xoo***
NaCl	41.40±21.34	0	58.33±18.85
$MgCl_2$	99.45±0.69	100	84.95±16.42
$CaCl_2$	53.1±11.22	55.55±21.99	0

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5.2.4 Steady state tryptophan fluorescence

Interaction of peptide with lipid vesicles was studied using steady-state tryptophan fluorescence. LCI_{22-47} contains three tryptophan residues *viz.* Trp23, Trp37, Trp44. In HEPES-buffered saline (HBS) and POPC:CHL SUVs, the peptide displays fluorescence emission maxima ~352 nm indicating solvent-exposed tryptophan residues (Figure 5.3). In the presence of POPE:POPG vesicles, however, distinct blue shift of around 8 nm with small enhancement in fluorescence intensity is observed suggesting binding of the peptide to lipid vesicles.

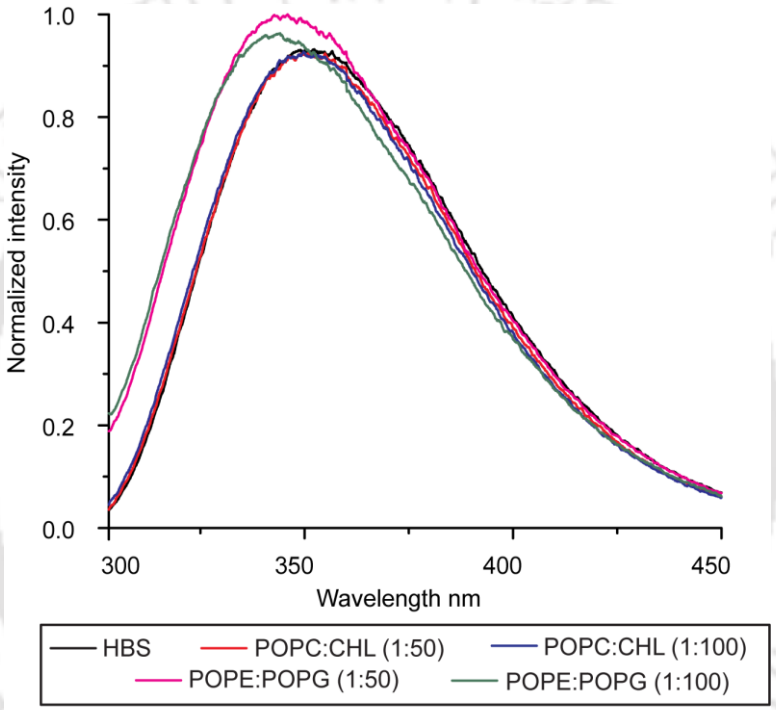

Figure 5.3 Tryptophan fluorescence emission spectra of LCI_{22-47} peptide. The spectra were recorded in the absence of lipid (black trace) and in the presence of SUVs such that the peptide/lipid ratios are 1:50 and 1:100.

5.2.5 Tryptophan fluorescence quenching

Accessibility of tryptophan side-chains to solvent was quantitatively determined by using aqueous quencher, acrylamide. Figure 5.4 shows the Stern-Volmer plots for the peptide in HBS, without and with SUVs. The plots obtained in HBS and with POPC:CHL SUVs show upward curvature, concave towards the F_0/F axis (panels A and B). An upward curvature indicates contribution from both static and dynamic quenching. Acrylamide is a well-established aqueous dynamic quencher for tryptophan fluorescence and is unlikely to bind

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to the LCI_{22-47} peptide, we attribute the upward curvature to the quenching sphere of action. Such apparent static quenching due to sphere of action has been observed for N-acetyltryptophanamide with acrylamide [228]. The modified Stern-Volmers equation that takes into account the apparent static quenching due to sphere of action is $F_0/F = (1+K_D[Q])e^{[Q]V}$, where, K_D is the dynamic quenching constant and V is the volume of the quenching sphere of action. Fitting of data with the modified Stern-Volmer equation gives the values of K_D and V. The quenching data obtained with POPE:POPG SUVs fits linearly and the Stern-Volmer constant thus obtained is considered as the dynamic quenching constant (panel C).

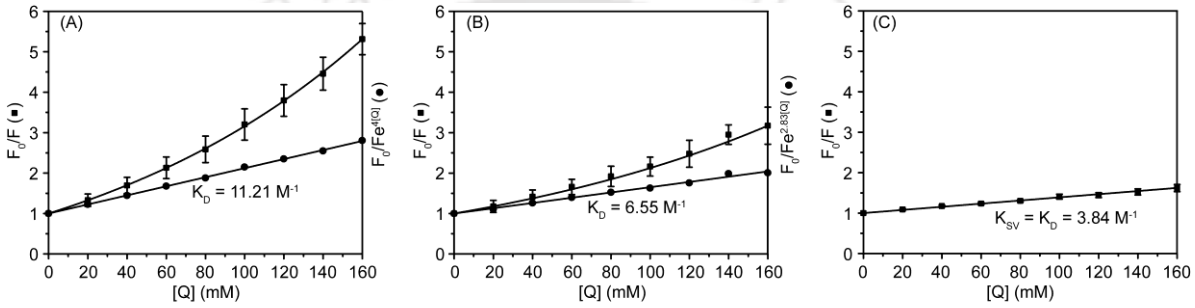

Figure 5.4 Stern-Volmer plots showing the tryptophan fluorescence quenching by acrylamide in LCI_{22-47} peptide in HBS (panel A), POPC:CHL SUVs (panel B), and POPE:POPG SUVs (panel C).

The net accessibility factor (NAF) is determined from the K_D values using the formula, NAF = K_D (with SUVs)/K_D (without SUVs). The K_D values obtained from figure 5.4 and the net accessibility factors (NAF) are shown in table 5.2.

Table 5.2 The Stern-Volmer constants and net accessibility factors for LCI_{22-47}

HBS		POPC:CHL		POPE:POPG	
K_{SV} (M^{-1})	NAF	K_{sv} (M^{-1})	NAF	K_{sv} (M^{-1})	NAF
11.21	1	6.55	0.58	3.84	0.34

5.2.6 Membrane permeabilization assay

Permeabilization of *E. coli* outer membrane was studied using NPN fluorescence as described in section 2.7, chapter 2. Polymyxin B (10 μg/ml) was used as a positive control in the assay. The data show ~70-78% membrane-permeabilization compared to the positive-control, polymyxin B suggesting membrane-permeabilization as one of the

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mechanisms of antibacterial action of LCI_{22-47} (Table 5.3) It is interesting to note that membrane-permeabilization is by and large concentration-independent in the 2–32 μM concentration range. Outer membrane permeabilization allows the AMPs a ready access to the inner membrane. An inner membrane permeabilization assay was carried out using voltage-sensitive dye, $diSC_3(5)$ as described in section 2.8, chapter 2. To my surprise, the peptide did not cause any inner membrane permeabilization up to a concentration of 16 μM. As the peptide is lethal to *E. coli* cells at a very low concentration of 2 μM, it is likely that the peptide uses a non-membrane-permeabilizing mechanism as well to kill the bacterium.

Table 5.3 *E. coli* outer membrane permeabilization by LCI_{22-47} peptide. The percentage NPN uptake was determined relative to that caused by 10 μg/ml polymyxin B.

LCI_{22-47} concentration (μM)	**Percentage NPN uptake**
2	69.51 ± 7.68
4	74.32 ± 15.34
8	78.30 ± 20.94
16	78.25 ± 20.08
32	72.60 ± 12.66

5.2.7 Circular dichroism

LCI_{22-47} displays a negative band around 215 nm in phosphate buffer alongside a positive band centered around 230 nm. The 215 nm band is suggestive of the β-sheet conformation. The peptide is unusually rich in aromatic residues and the 230 nm band is assigned to the aromatic clusters in the folded peptide. The peptide also displays a negative band around 205 nm suggesting the presence of unordered conformation as well. The peptide structure is, by and large, same in the presence of POPC:CHL vesicles suggesting little interaction with lipids, if any. In POPE:POPG vesicles, however, a completely different CD signature is obtained; the peptide shows a broad negative band centered around 218 nm suggesting a β-sheet conformation. Another interesting feature to note is the complete absence of the 230 nm positive band suggesting large scale structural rearrangements in the peptide structure upon lipid binding. In TFE, the peptide takes up an α-helical conformation

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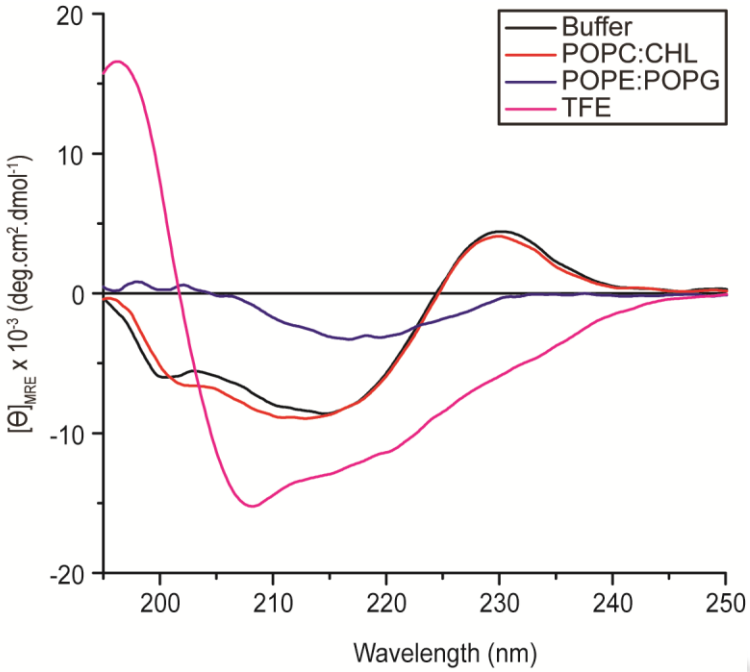

Figure 5.5 Circular dichroism spectra of LCI_{22-47}. Spectra recorded in 10 mM phosphate buffer, pH 7.4 having 100 mM NaCl and 0.1 mM EDTA without and with SUVs.

5.2.8 Hemolytic assay

Toxicity of LCI_{22-47} to human erythrocytes was tested at 2, 8, and 32 μM peptide concentrations as described in section 2.13, chapter 2. Human erythrocytes were incubated with peptide for 1 hour and percentage lysis determined. At 2 and 8 μM peptide concentrations, no detectable hemolysis was obtained. At 32 μM peptide concentration, however, little hemolysis (0.5%) was observed.

5.2.9 FESEM analysis

FESEM images of the LCI_{22-47}-treated bacteria are shown in figure 5.6. The peptide caused extensive damage to all the three bacteria wherein the bacterial cell integrity is severely compromised. Such disruption of bacterial cells supports the bactericidal rather than bacteriostatic activity of the peptide.

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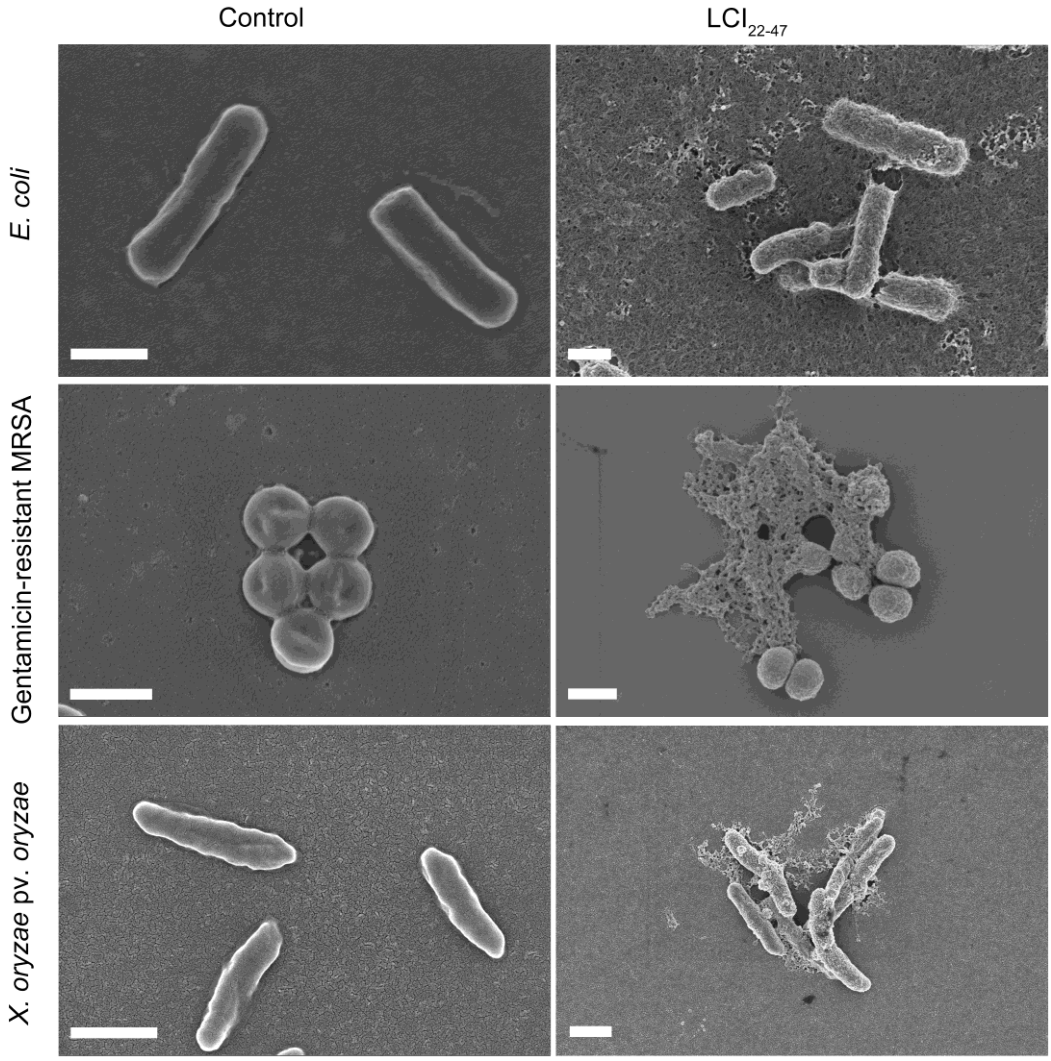

Figure 5.6 FESEM micrographs of LCI$_{22-47}$ peptide-treated bacteria.

5.3 Conclusion

Identification of shorter peptides without a loss in activity is a cost-effective measure in the developing such drugs. LCI, the 47-residue peptide produced by *B. subtilis*, inhibits plant pathogens; the peptide is reported to inhibit 50% growth of *Xanthomonas* at a concentration of 0.81 μM [64]. The peptide, however, is reported to be inactive against *E. coli* [63]. Here I show that the C-terminal stretch of peptide, LCI$_{22-47}$ possesses strong antibacterial activity not only against plant pathogen, *Xoo* but againt *E. coli* and gentamicin-resistant MRSA as well. All the three bacteria are completely killed with lethal concentrations within 4 μM. Interestingly, the activity against *E. coli* (2 μM lethal concentration) is better than that against *Xoo* (4 μM lethal concentration). In addition to that, the peptide retains substantial activity against *E. coli* even in the presence of salt and divalent cations. The activity of the

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peptide at its lethal concentrations against *Xoo* and gentamicin-resistant MRSA, however, is completely lost at 2 mM $CaCl_2$ and 100 mM NaCl, respectively. This loss in activity, however, does not imply complete inactivity of the peptide; it implies that the peptide displays antibacterial activity at higher concentrations. The peptide causes *E. coli* outer membrane permeabilization, suggesting membrane-permeabilization as one of the mechanisms underlying antibacterial activity. The mechanism of LCI antimicrobial action is not known. The structure of LCI suggests that the peptide is not amphipathic, an aspect similar to rabbit kidney defensin, RK-1 [229]. RK-1, like rabbit neutrophil defensins, is proposed to form short-lived pores in liposomes composed of *E. coli* membrane lipids [229, 230]. It is interesting to note that LCI_{22-47} causes ~70% *E. coli* outer membrane permeabilization at 2 μM concentration. Higher concentrations up to 32 μM did not cause any appreciable increase in membrane-permeabilization. In addition to that, the peptide failed to cause detectable inner membrane permeabilization. Such behavior could arise due to following possibilities: (i) the formation of transient pores that permit only small molecules to pass through thereby denying the peptide molecules access to inner membrane or (ii) translocation of the peptides across the inner membrane without disrupting it. The data suggest that membrane-permeabilization may not be the sole mechanism of bacterial killing caused by LCI_{22-47}. Further investigations with LCI and LCI_{22-47} are necessary to obtain mechanistic insights into their antimicrobial activity. Nonetheless, the LCI_{22-47} fragment is a promising AMP against both Gram-negative and Gram-positive bacteria.

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CHAPTER 6

Conclusion, Discussion, and Possibilities

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Abuse of antibiotics has led to an alarming situation wherein many of the clinical strains have developed resistance against multiple antibiotics available. Emerging resistance to the existing antibiotics together with the very slow discovery of new class of antibiotics is one of the major health threats today. There is an urgent need for the new class of antimicrobials and peptides are being looked at with great expectations [231]. AMPs are natural defense molecules found in all life forms. They have sustained the evolutionary pressure without causing any significant antimicrobial resistance making them the molecules of choice for developing the new generation of antimicrobials. It is imperative to design antimicrobials that are cost-effective and possess broad-spectrum antimicrobial activity without instigating any toxic effect to the host. Naturally occurring AMPs are often 20-50 residues long, and the high cost of peptide synthesis is one of the major obstacles in developing peptides as antibiotics [16, 232]. The focus, therefore, is shifting towards designing and developing shorter AMPs [71]. This thesis is concerned with antimicrobial properties of the peptides derived from proteins of bacterial origin.

Chapter 1 is an introduction to AMPs alongside literature review. Chapter 2 contains the materials and methodologies employed. Chapters 3 and 4 discuss the antimicrobial potential of short peptides (≤13 residues) derived from membrane-interacting stretches of *E. coli* proteins MreB and FtsA, respectively. In parent proteins, these stretches fold into amphipathic helices, driving their tethering to the cytoplasmic membrane. The selected peptides possess a net charge of +3 to +5 at neutral pH and were predicted to be membrane-binding ones by Heliquest analyses. All the peptides turned out to be potent antimicrobials; the MreB-derived peptides killed both Gram-negative and Gram-positive bacteria at comparable concentrations. The tryptophan-extended peptides *i.e.* $MreB_{1\text{-}9}$, extended by a Trp residue at N-terminus turned out to be highly active. Gram-negative bacteria namely *E. coli, P. aeruginosa, S. enterica*; Gram-positive bacterium *S. aureus*; and opportunistic yeast *C. albicans* were killed with lethal concentrations within 10 μM. Like MreB-derived peptides, the FtsA-derived peptides also turned out to be active against all these organisms. The peptides, however, displayed selectivity towards Gram-negative bacteria; the 13-residue peptides displayed MIC of 4 μM or better against *E. coli*, *P. aeruginosa*, and *S. enterica* whereas an MIC of 32 μM was obtained against gentamicin-resistant MRSA. The 11-residue peptides were little less active with MICs ranging from 2-

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16 μM but displayed similar selectivity towards Gram-negative bacteria. The peptides displayed potent activity against *C. albicans* as well. This selectivity towards Gram-negative bacteria can be attributed to the physicochemical properties of peptides. Malanovic and Lohner have recently carried out statistical analysis with known AMPs to understand the AMP selectivity towards bacteria [233]. The analysis indicates a correlation between the peptide hydrophobicity and bacterial selectivity. Peptides that act against Gram-negative bacteria are less hydrophobic (≤50% hydrophobic residues). The most potent, Trp-extended MreB-derived peptides possess high mean hydrophobicity (<H>) of 0.7 with 60% of the residues hydrophobic and a moderate mean hydrophobic moment (<μH>) of 0.523. The FtsA-derived peptides, on the other hand, possess low or moderate mean hydrophobicity (≤0.46) with 45% residues hydrophobic for 11-residue peptides and only 38% residues hydrophobic for 13-residue peptides. The low hydrophobicity of the FtsA-derived peptides could be responsible for imparting them the selectivity towards Gram-negative bacteria.

The cell wall of Gram-positive bacteria contains lipoteichoic acid whereas Gram-negative bacteria contain lipopolysaccharide (LPS) as a major component of their outer membrane. These structures are characterized by a variable number of phosphate groups and sugars. These anionic molecules are bridged by the divalent cations *viz.* Ca^{2+} and Mg^{2+} [1]. Growth media deficient in these cations might compromise bacterial cell wall integrity thereby affecting their susceptibility to AMPs. As binding of AMPs to negatively charged bacterial surface is mediated through electrostatic interaction, the presence of salt can interfere with this binding. Diminished activity in the presence of salt and divalent cations has indeed been reported for many AMPs [122, 234-237]. It was therefore imperative to test the activity of the peptides in the presence of salts and divalent cations at physiologically relevant concentrations. The sensitivity of peptides to salt and divalent cations was, therefore assessed by determining their activity in the presence of 100 mM NaCl, 1 mM $MgCl_2$, and 2 mM $CaCl_2$. The activity of peptides was antagonised by salt and divalent cations in a species-dependent manner as detailed in chapters 3 and 4. Greater than 50% activity of $MreB_{1-9}$ and 100% activity of Ac-W-$MreB_{1-9}$ against *E. coli* are the highlights of chapter 3; *E. coli*-derived peptides killed *E. coli*. Ac-W-$MreB_{1-9}$ peptide happens to be another highlight of the study; the peptide retains 80% or more activity against all the bacteria tested and >40% activity against *C. albicans* in the presence of salt and divalent cations. This peptide,

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therefore, turns out to be a promising short AMP. Salt and divalent cations antagonize the activity of the FtsA-derived peptides as well. Some of the highlights of the FtsA-derived peptides include: (i) MICs of 11 and 13-residue peptides are within 8 μM against *E. coli* and (ii) Ac-FtsA11 and the 13-residue peptides display MICs within 16 μM against Gram-negative bacteria in the presence of salt and divalent cations. None of the peptides resulted in any appreciable hemolysis at their lethal concentrations or MICs.

As all these peptides possess a net positive charge at neutral pH and are expected to kill the microbes by permeabilizing their membranes like other cationic AMPs reported in the literature, their interaction with model membranes was studied. As anticipated, all the peptides display preferential binding to negatively charged lipid vesicles as ascertained by Trp fluorescence and quenching studies. Binding to lipid vesicles alone, however, does not imply membrane permeabilization as the mechanism of killing. Permeabilization of the membrane was assayed using *E. coli* as the model organism; all the potent peptides caused both inner and outer membrane permeabilization suggesting this as one of the mechanisms of killing. These data support the hypothesis that a short membrane-binding stretch of a bacterial protein can kill the very same bacteria. AMPs derived from membrane-binding stretches of pathogenic organism's proteins, therefore, could be an interesting antimicrobial strategy. The peptides could kill the very same microbe by permeabilizing the membrane and possibly by disturbing the homeostasis if they bind to the intracellular targets of the parent protein. In addition to that, the peptides could combat other microbes as well through usual membrane permeabilization mechanism as is observed for the peptides derived from *E. coli* MreB and FtsA proteins; in addition to efficiently inhibiting *E. coli*, the peptides inhibit *P. aeruginosa*, *S. enterica*, gentamicin-resistant MRSA and *C. albicans* as well. Compared to the drugs that target specific protein receptors, development of resistance against membrane targeting molecules is less common. Moreover, it would be interesting to test if self-like peptide sequences can escape the proteolytic cleavage by bacterial proteases.

Chapter 5 discusses the antimicrobial efficacy of LCI_{22-47} peptide fragment derived from the C-terminal region of LCI protein from *Bacillus subtilis*. The native protein was reported to possess antimicrobial activity against plant pathogens but was inactive against *E. coli*.

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The 26-residue C-terminal fragment, however, turned out to be a potent antimicrobial with minimum lethal concentration of 2 μM against *E. coli* and 4 μM against gentamicin-resistant MRSA and *Xanthomonas oryzae.* In the presence of salts and divalent cations, the peptide's activity was compromised. However, at double the lethal concentration, peptide displayed significant antimicrobial activity. Moreover, membrane binding studies have also shown peptide's ability to interact and permeabilize the bacterial outer membrane. Permeabilization of inner–membrane, however, was not observed even at a higher concentration of peptide suggesting possibly a non-membrane permeabilizing mode of action.

The new antibiotics that entered medicine in the last five decades were all against Gram-positive bacteria. Largely due to the lack of new antibiotics that could combat Gram-negative bacteria, Gram-negative bacteremia has become a serious health issue [16, 238, 239]. Gram-negative pathogens are becoming resistant to nearly all the antibiotics available, creating situations indicative of the pre-antibiotic era [240]. The recently discovered antibiotics, teixobactin and malacidins are also active against Gram-positive bacteria [204, 241]. In addition to that, Gram-positive bacteria, in general, have a higher content of negatively charged lipids and most AMPs reported in the literature show better activity against Gram-positive bacteria. Peptides possessing activity against Gram-negative bacteria, therefore, need special mention. The most serious Gram-negative bacterial infections in healthcare include *Klebsiella pneumoniae*, *P. aeruginosa*, and *Acinetobacter* [240, 242]. MDR Gram-negative pathogens like beta-lactamase-producing *E. coli* and *Neisseria gonorrhoeae* resistant to fluoroquinolones, penicillin, and tetracycline are becoming prevalent in the population [240]. *P. aeruginosa* turns out to be one of the most common human pathogens. It is one of the two most common pathogens causing nosocomial pneumonia, *S. aureus* being the other one. *P. aeruginosa* is an opportunistic pathogen and the serious Pseudomonal infections are often hospital-acquired [243]. It happens to be the most commonly isolated pathogen from patients that are hospitalized for long durations especially the ones that have compromised immune system. It is an intrinsically resistant bacterium to many antibiotics and is capable of acquiring resistance to multiple antibiotics [238, 243]. It is interesting to note that the MreB-derived peptides kill both Gram-positive

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and Gram-negative bacteria equally efficiently. FtsA-derived peptides, on the other hand, display selectivity towards Gram-negative bacteria.

S. aureus is one of the most clinically-relevant Gram-positive bacteria. It is the most common bacterium present on human skin. It is considered an opportunistic pathogen, but more aggressive strains have also evolved. *S. aureus* infections could be easily treated using Penicillin in 1940s [244]. Subsequently, *S. aureus* developed resistance against penicillin and could be treated with a penicillin analog, methicillin. A methicillin-resistant *S. aureus* strain was identified in 1960s and this was the birth of difficult to treat methicillin-resistant *S. aureus* (MRSA) [245]. It is arguably the most important hospital-acquired human pathogen. Although historically a hospital-acquired infection, community-acquired MRSA strains have emerged and are now epidemic in United States. It is very urgent to come up with new antimicrobials to treat MRSA infections. MreB-derived peptides displayed excellent activity against gentamicin-resistant MRSA; all the four peptides retained ~90% or more activity in the presence of salt and divalent cations. *Candida* is a clinically important fungus, and *C. albicans* happens to be the most common species causing invasive candidiasis [219]. All the four MreB-derived peptides and the 11 and 13-residue FtsA-derived peptides efficiently kill *C. albicans* and retain substantial activity in the presence of salt and divalent cations.

Tuberculosis (TB), continues to be one of the most dreadful communicable diseases. TB is caused by *Mycobacterium tuberculosis* (MTB), a pathogenic bacterium that replicates inside host phagocytic cells and can evade host immune responses. Several drugs have been developed to tackle TB, but the emergence of multi-drug resistant (MDR) and extensively drug resistant (XDR) strains has kept the threat on [246]. More than 1.5 million people are estimated to die every year due to tuberculosis [247]. The difficulty in treating TB is largely conferred by the complex cell-wall structure of MTB. A complex cell wall core surrounds the inner membrane in MTB. The "cell wall core" is characterized by a peptidoglycan layer covalently attached to arabinogalactan that in turn is covalently attached to the mycolic acids to form the so-called mAGP (mycolyl-Arabinogalactan-Peptidoglycan) complex [248]. The cell wall acts as a barrier to drug molecules; the outer membrane is particularly impermeable to small polar molecules with permeability 10-1000 times lower than the

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outer membrane of Gram-negative bacteria [249]. It would be interesting to extend the concept of membrane-interacting self-sequences to combat *M. tuberculosis*. We tested, as a preliminary study, the anti-mycobacterial properties of *E. coli* MreB and FtsA-derived peptides against *M. smegmatis*, a non-pathogenic and fast-growing mycobacterium strain.

The *E. coli* MreB and FtsA-derived peptides, that turned out to be the most potent ones against *E. coli* and other Gram-negative bacteria, *i.e.* W-MreB$_{1-9}$, Ac-W-MreB$_{1-9}$, FtsA11, Ac-FtsA11, FtsA13, and Ac-FtsA13 were tested for their anti-mycobacterial potential. The MIC was determined using the broth microdilution method as described in section 2.10.3, chapter 2. All the peptides turn out to be active against *M. smegmatis*. The 13-residue FtsA-derived peptides happen to be the most potent ones (MIC = 2 μM). Other four peptides also turned to be potent antimycobacterials (Table 6.1). The sensitivity of anti-mycobacterial activity to salt and divalent cations was assessed by determining the MIC values in the presence of 100 mM NaCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$ (Table 6.1). All but one of the peptides retained substantial activity (MIC ≤8 μM) in the presence of salt and divalent cations. MIC of WMreB$_{1-9}$ was considerably compromised in NaCl resulting in an MIC of 32 μM.

Table 6.1 MIC of the MreB and FtsA-derived peptides against *M. smegmatis*.

Peptide	**Minimum inhibitory concentration (μM)**			
	M. smegmatis			
	Absence of salts	NaCl	$MgCl_2$	$CaCl_2$
W-MreB$_{1-9}$	8	32	8	8
Ac-WMreB$_{1-9}$	4	8	4	8
FtsA11	4	8	4	4
Ac-FsA11	4	4	4	4
FtsA13	2	8	2	4
Ac-FtsA13	2	4	2	2

FESEM images of peptide-treated *M. smegmatis* indicate large-scale disruption of bacteria (Figure 6.1) indicating membrane-permeabilization as the possible mechanism of peptide action.

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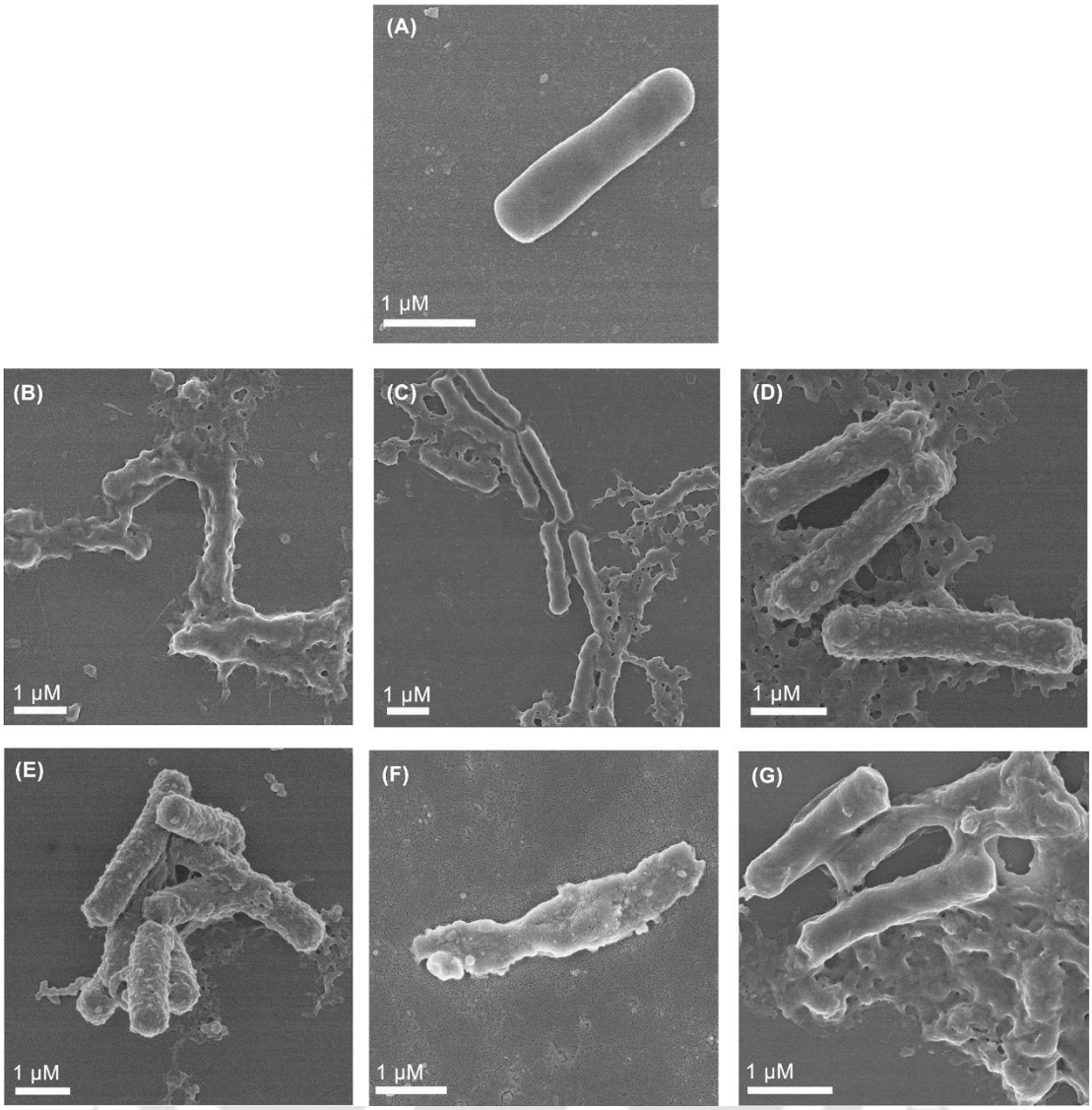

Figure 6.1 FESEM micrographs of peptide-treated *M. smegmatis*, (A) Control, (B) FtsA11, (C) Ac-FtsA11, (D) FtsA13, (E) Ac-FtsA13, (F) W-MreB$_{1-9}$, and (G) Ac-W-MreB$_{1-9}$.

These data are very promising and encouraging to design novel AMPs from the membrane-binding regions of proteins from *M. tuberculosis* and other difficult to treat microbes. In addition, following open questions would be interesting to address:

1. What happens to the peptides at concentrations much lower than their lethal concentrations or MICs?
2. Do peptides get translocated across the membrane at sub-lethal concentrations?
3. If somehow put inside the bacterial cytosol, would such a peptide compete with the parent protein for its targets including membrane?

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4. Can bacteria selectively pump out or degrade a peptide that is identical or very similar to its protein stretch? If so, how?

5. Do bacteria use such peptide stretches to combat the invading bacterial species by somehow protecting themselves or in a suicidal fashion wherein bacterial cells lyse to release these molecules to combat the invading bacteria?

6. Will a bacterium come up with a resistant-strategy to counter a peptide that is identical/similar to its peptide stretch?

7. How stable are the peptides in human serum?

To conclude, the membrane-binding stretches and possibly other amphipathic stretches in the microbial proteins can be a great source of novel antimicrobial peptides to combat the very same pathogen.

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REFERENCES

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1. Zasloff, M., *Antimicrobial peptides of multicellular organisms.* Nature, 2002. **415**(6870): p. 389-95.
2. Cederlund, A., G.H. Gudmundsson, and B. Agerberth, *Antimicrobial peptides important in innate immunity.* FEBS J, 2011. **278**(20): p. 3942-51.
3. Mishra, B. and G. Wang, *Ab initio design of potent anti-MRSA peptides based on database filtering technology.* J Am Chem Soc, 2012. **134**(30): p. 12426-9.
4. Fan, L., et al., *DRAMP: a comprehensive data repository of antimicrobial peptides.* Sci Rep, 2016. **6**: p. 24482.
5. Jenssen, H., P. Hamill, and R.E. Hancock, *Peptide antimicrobial agents.* Clin Microbiol Rev, 2006. **19**(3): p. 491-511.
6. Huang, Y., J. Huang, and Y. Chen, *Alpha-helical cationic antimicrobial peptides: relationships of structure and function.* Protein Cell, 2010. **1**(2): p. 143-52.
7. Giangaspero, A., L. Sandri, and A. Tossi, *Amphipathic alpha helical antimicrobial peptides.* Eur J Biochem, 2001. **268**(21): p. 5589-600.
8. Aminov, R.I., *A brief history of the antibiotic era: lessons learned and challenges for the future.* Front Microbiol, 2010. **1**: p. 134.
9. Fair, R.J. and Y. Tor, *Antibiotics and bacterial resistance in the 21st century.* Perspect Medicin Chem, 2014. **6**: p. 25-64.
10. Hancock, R.E. and R. Lehrer, *Cationic peptides: a new source of antibiotics.* Trends Biotechnol, 1998. **16**(2): p. 82-8.
11. Czaplewski, L., et al., *Alternatives to antibiotics-a pipeline portfolio review.* Lancet Infect Dis, 2016. **16**(2): p. 239-51.
12. Reddy, K.V., R.D. Yedery, and C. Aranha, *Antimicrobial peptides: premises and promises.* Int J Antimicrob Agents, 2004. **24**(6): p. 536-47.
13. Tossi, A., L. Sandri, and A. Giangaspero, *Amphipathic, alpha-helical antimicrobial peptides.* Biopolymers, 2000. **55**(1): p. 4-30.
14. Pushpanathan, M., P. Gunasekaran, and J. Rajendhran, *Antimicrobial peptides: versatile biological properties.* Int J Pept, 2013. **2013**: p. 675391.
15. Fjell, C.D., et al., *Designing antimicrobial peptides: form follows function.* Nat Rev Drug Discov, 2011. **11**(1): p. 37-51.
16. Marr, A.K., W.J. Gooderham, and R.E. Hancock, *Antibacterial peptides for therapeutic use: obstacles and realistic outlook.* Curr Opin Pharmacol, 2006. **6**(5): p. 468-72.
17. Hoskin, D.W. and A. Ramamoorthy, *Studies on anticancer activities of antimicrobial peptides.* Biochim Biophys Acta, 2008. **1778**(2): p. 357-75.
18. Felicio, M.R., et al., *Peptides with Dual Antimicrobial and Anticancer Activities.* Front Chem, 2017. **5**: p. 5.
19. Luo, Y., et al., *The Naturally Occurring Host Defense Peptide, LL-37, and Its Truncated Mimetics KE-18 and KR-12 Have Selected Biocidal and Antibiofilm Activities Against Candida albicans, Staphylococcus aureus, and Escherichia coli In vitro.* Front Microbiol, 2017. **8**: p. 544.
20. Gordya, N., et al., *Natural antimicrobial peptide complexes in the fighting of antibiotic resistant biofilms: Calliphora vicina medicinal maggots.* PLoS One, 2017. **12**(3): p. e0173559.
21. Gordon, Y.J., E.G. Romanowski, and A.M. McDermott, *A review of antimicrobial peptides and their therapeutic potential as anti-infective drugs.* Curr Eye Res, 2005. **30**(7): p. 505-15.
22. Hancock, R.E. and D.S. Chapple, *Peptide antibiotics.* Antimicrob Agents Chemother, 1999. **43**(6): p. 1317-23.
23. Wang, G., X. Li, and Z. Wang, *APD3: the antimicrobial peptide database as a tool for research and education.* Nucleic Acids Res, 2016. **44**(D1): p. D1087-93.
24. Yeaman, M.R. and N.Y. Yount, *Mechanisms of antimicrobial peptide action and resistance.* Pharmacol Rev, 2003. **55**(1): p. 27-55.

TH-2072_126106010

25. Toke, O., *Antimicrobial peptides: new candidates in the fight against bacterial infections.* Biopolymers, 2005. **80**(6): p. 717-35.
26. Mahlapuu, M., et al., *Antimicrobial Peptides: An Emerging Category of Therapeutic Agents.* Front Cell Infect Microbiol, 2016. **6**: p. 194.
27. Tareq, F.S., et al., *Gageotetrins A–C, Noncytotoxic Antimicrobial Linear Lipopeptides from a Marine Bacterium Bacillus subtilis.* Organic Letters, 2014. **16**(3): p. 928-931.
28. Bangalore, N., et al., *Identification of the primary antimicrobial domains in human neutrophil cathepsin G.* J Biol Chem, 1990. **265**(23): p. 13584-8.
29. Liu, Y.Q., et al., *Purification of a novel antibacterial short peptide in earthworm Eisenia foetida.* Acta Biochim Biophys Sin (Shanghai), 2004. **36**(4): p. 297-302.
30. Zhu, X., et al., *Purification and characterisation of plantaricin ZJ008, a novel bacteriocin against Staphylococcus spp. from Lactobacillus plantarum ZJ008.* Food Chem, 2014. **165**: p. 216-23.
31. Fleming, A. and V.D. Allison, *Observations on a Bacteriolytic Substance ("Lysozyme") Found in Secretions and Tissues.* British journal of experimental pathology, 1922. **3**(5): p. 252-260.
32. Nakatsuji, T. and R.L. Gallo, *Antimicrobial peptides: old molecules with new ideas.* J Invest Dermatol, 2012. **132**(3 Pt 2): p. 887-95.
33. Brown, K., *The history of penicillin from discovery to the drive to production.* Pharm Hist (Lond), 2004. **34**(3): p. 37-43.
34. Hasper, H.E., et al., *An alternative bactericidal mechanism of action for lantibiotic peptides that target lipid II.* Science, 2006. **313**(5793): p. 1636-7.
35. Dubos, R.J., *Studies on a Bactericidal Agent Extracted from a Soil Bacillus : I. Preparation of the Agent. Its Activity in Vitro.* J Exp Med, 1939. **70**(1): p. 1-10.
36. Dubos, R.J., *Studies on a Bactericidal Agent Extracted from a Soil Bacillus : Ii. Protective Effect of the Bactericidal Agent against Experimental Pneumococcus Infections in Mice.* J Exp Med, 1939. **70**(1): p. 11-7.
37. Van Epps, H.L., *Rene Dubos: unearthing antibiotics.* J Exp Med, 2006. **203**(2): p. 259.
38. Fernandez de Caleya, R., et al., *Susceptibility of phytopathogenic bacteria to wheat purothionins in vitro.* Appl Microbiol, 1972. **23**(5): p. 998-1000.
39. Velkov, T., et al., *Pharmacology of polymyxins: new insights into an 'old' class of antibiotics.* Future Microbiol, 2013. **8**(6): p. 711-24.
40. Arnold, T.M., G.N. Forrest, and K.J. Messmer, *Polymyxin antibiotics for gram-negative infections.* Am J Health Syst Pharm, 2007. **64**(8): p. 819-26.
41. Fennell, J.F., W.H. Shipman, and L.J. Cole, *Antibacterial action of a bee venom fraction (melittin) against a penicillin-resistant staphylococcus and other microorganisms. USNRDL-TR-67-101.* Res Dev Tech Rep, 1967: p. 1-13.
42. Wu, Q., J. Patocka, and K. Kuca, *Insect Antimicrobial Peptides, a Mini Review.* 2018. **10**(11).
43. Gajski, G. and V. Garaj-Vrhovac, *Melittin: a lytic peptide with anticancer properties.* Environ Toxicol Pharmacol, 2013. **36**(2): p. 697-705.
44. Simmaco, M., G. Kreil, and D. Barra, *Bombinins, antimicrobial peptides from Bombina species.* Biochim Biophys Acta, 2009. **1788**(8): p. 1551-5.
45. Gran, L., *On the effect of a polypeptide isolated from "Kalata-Kalata" (Oldenlandia affinis DC) on the oestrogen dominated uterus.* Acta Pharmacol Toxicol (Copenh), 1973. **33**(5): p. 400-8.
46. Tam, J.P., et al., *An unusual structural motif of antimicrobial peptides containing end-to-end macrocycle and cystine-knot disulfides.* Proc Natl Acad Sci U S A, 1999. **96**(16): p. 8913-8.
47. Hultmark, D., et al., *Insect immunity. Purification and properties of three inducible bactericidal proteins from hemolymph of immunized pupae of Hyalophora cecropia.* Eur J Biochem, 1980. **106**(1): p. 7-16.

TH-2072_126106010

48. Steiner, H., et al., *Sequence and specificity of two antibacterial proteins involved in insect immunity.* Nature, 1981. **292**(5820): p. 246-8.
49. Selsted, M.E., et al., *Primary structures of MCP-1 and MCP-2, natural peptide antibiotics of rabbit lung macrophages.* Journal of Biological Chemistry, 1983. **258**(23): p. 14485-14489.
50. Selsted, M.E., D. Szklarek, and R.I. Lehrer, *Purification and antibacterial activity of antimicrobial peptides of rabbit granulocytes.* Infection and Immunity, 1984. **45**(1): p. 150-154.
51. Selsted, M.E., et al., *Primary structures of six antimicrobial peptides of rabbit peritoneal neutrophils.* Journal of Biological Chemistry, 1985. **260**(8): p. 4579-4584.
52. Ganz, T., et al., *Defensins. Natural peptide antibiotics of human neutrophils.* J Clin Invest, 1985. **76**(4): p. 1427-35.
53. Selsted, M.E., et al., *Primary structures of three human neutrophil defensins.* The Journal of Clinical Investigation, 1985. **76**(4): p. 1436-1439.
54. Zasloff, M., *Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor.* Proc Natl Acad Sci U S A, 1987. **84**(15): p. 5449-53.
55. Nakamura, T., et al., *Tachyplesin, a class of antimicrobial peptide from the hemocytes of the horseshoe crab (Tachypleus tridentatus). Isolation and chemical structure.* J Biol Chem, 1988. **263**(32): p. 16709-13.
56. Oppenheim, F.G., et al., *Histatins, a novel family of histidine-rich proteins in human parotid secretion. Isolation, characterization, primary structure, and fungistatic effects on Candida albicans.* J Biol Chem, 1988. **263**(16): p. 7472-7.
57. Diamond, G., et al., *Tracheal antimicrobial peptide, a cysteine-rich peptide from mammalian tracheal mucosa: peptide isolation and cloning of a cDNA.* Proc Natl Acad Sci U S A, 1991. **88**(9): p. 3952-6.
58. Agerberth, B., et al., *Amino acid sequence of PR-39. Isolation from pig intestine of a new member of the family of proline-arginine-rich antibacterial peptides.* Eur J Biochem, 1991. **202**(3): p. 849-54.
59. Cowland, J.B., A.H. Johnsen, and N. Borregaard, *hCAP-18, a cathelin/pro-bactenecin-like protein of human neutrophil specific granules.* FEBS Lett, 1995. **368**(1): p. 173-6.
60. Gudmundsson, G.H., et al., *The human gene FALL39 and processing of the cathelin precursor to the antibacterial peptide LL-37 in granulocytes.* Eur J Biochem, 1996. **238**(2): p. 325-32.
61. Nielsen, K.L., et al., *Role of urinary cathelicidin LL-37 and human beta-defensin 1 in uncomplicated Escherichia coli urinary tract infections.* Infect Immun, 2014. **82**(4): p. 1572-8.
62. Tang, Y.Q., et al., *A cyclic antimicrobial peptide produced in primate leukocytes by the ligation of two truncated alpha-defensins.* Science, 1999. **286**(5439): p. 498-502.
63. Liu, J.-Y., N.-S. Pan, and Z.-L. Chen, *Characterization of an anti-rice bacterial blight polypeptide LCI.* Rice Genetic Newsletter, 1990. **7**: p. 151-154.
64. Gong, W., et al., *Solution structure of LCI, a novel antimicrobial peptide from Bacillus subtilis.* Biochemistry, 2011. **50**(18): p. 3621-7.
65. Holthausen, D.J., et al., *An Amphibian Host Defense Peptide Is Virucidal for Human H1 Hemagglutinin-Bearing Influenza Viruses.* Immunity, 2017. **46**(4): p. 587-595.
66. Brogden, K.A., *Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?* Nat Rev Microbiol, 2005. **3**(3): p. 238-50.
67. Gennaro, R. and M. Zanetti, *Structural features and biological activities of the cathelicidin-derived antimicrobial peptides.* Biopolymers, 2000. **55**(1): p. 31-49.
68. Hancock, R.E., *Peptide antibiotics.* Lancet, 1997. **349**(9049): p. 418-22.
69. Hancock, R.E. and A. Patrzykat, *Clinical development of cationic antimicrobial peptides: from natural to novel antibiotics.* Curr Drug Targets Infect Disord, 2002. **2**(1): p. 79-83.

TH-2072_126106010

70. Pasupuleti, M., A. Schmidtchen, and M. Malmsten, *Antimicrobial peptides: key components of the innate immune system.* Crit Rev Biotechnol, 2012. **32**(2): p. 143-71.
71. Seo, M.D., et al., *Antimicrobial peptides for therapeutic applications: a review.* Molecules, 2012. **17**(10): p. 12276-86.
72. Sipos, D., M. Andersson, and A. Ehrenberg, *The structure of the mammalian antibacterial peptide cecropin P1 in solution, determined by proton-NMR.* Eur J Biochem, 1992. **209**(1): p. 163-9.
73. Agerberth, B., et al., *FALL-39, a putative human peptide antibiotic, is cysteine-free and expressed in bone marrow and testis.* Proc Natl Acad Sci U S A, 1995. **92**(1): p. 195-9.
74. Marion, D., M. Zasloff, and A. Bax, *A two-dimensional NMR study of the antimicrobial peptide magainin 2.* FEBS Lett, 1988. **227**(1): p. 21-6.
75. Raghuraman, H. and A. Chattopadhyay, *Melittin: a membrane-active peptide with diverse functions.* Biosci Rep, 2007. **27**(4-5): p. 189-223.
76. Harris, F., S.R. Dennison, and D.A. Phoenix, *Anionic antimicrobial peptides from eukaryotic organisms.* Curr Protein Pept Sci, 2009. **10**(6): p. 585-606.
77. Lehrer, R.I. and T. Ganz, *Endogenous Vertebrate Antibiotics.* Annals of the New York Academy of Sciences, 1996. **797**(1): p. 228-239.
78. Matsuzaki, K., et al., *Membrane permeabilization mechanisms of a cyclic antimicrobial peptide, tachyplesin I, and its linear analog.* Biochemistry, 1997. **36**(32): p. 9799-806.
79. Rao, A.G., *Conformation and antimicrobial activity of linear derivatives of tachyplesin lacking disulfide bonds.* Arch Biochem Biophys, 1999. **361**(1): p. 127-34.
80. Ganz, T., *Defensins: antimicrobial peptides of innate immunity.* Nat Rev Immunol, 2003. **3**(9): p. 710-20.
81. Wei, G., et al., *Through the looking glass, mechanistic insights from enantiomeric human defensins.* J Biol Chem, 2009. **284**(42): p. 29180-92.
82. Hwang, P.M., et al., *Three-dimensional solution structure of lactoferricin B, an antimicrobial peptide derived from bovine lactoferrin.* Biochemistry, 1998. **37**(12): p. 4288-98.
83. Fahrner, R.L., et al., *Solution structure of protegrin-1, a broad-spectrum antimicrobial peptide from porcine leukocytes.* Chem Biol, 1996. **3**(7): p. 543-50.
84. Trabi, M. and D.J. Craik, *Circular proteins--no end in sight.* Trends Biochem Sci, 2002. **27**(3): p. 132-8.
85. Martinez-Bueno, M., et al., *Determination of the gene sequence and the molecular structure of the enterococcal peptide antibiotic AS-48.* J Bacteriol, 1994. **176**(20): p. 6334-9.
86. Gonzalez, C., et al., *Bacteriocin AS-48, a microbial cyclic polypeptide structurally and functionally related to mammalian NK-lysin.* Proc Natl Acad Sci U S A, 2000. **97**(21): p. 11221-6.
87. Galvez, A., et al., *Purification and amino acid composition of peptide antibiotic AS-48 produced by Streptococcus (Enterococcus) faecalis subsp. liquefaciens S-48.* Antimicrob Agents Chemother, 1989. **33**(4): p. 437-41.
88. Saether, O., et al., *Elucidation of the primary and three-dimensional structure of the uterotonic polypeptide kalata B1.* Biochemistry, 1995. **34**(13): p. 4147-58.
89. Gran, L., F. Sandberg, and K. Sletten, *Oldenlandia affinis (R&S) DC. A plant containing uteroactive peptides used in African traditional medicine.* J Ethnopharmacol, 2000. **70**(3): p. 197-203.
90. Trabi, M., H.J. Schirra, and D.J. Craik, *Three-dimensional structure of RTD-1, a cyclic antimicrobial defensin from Rhesus macaque leukocytes.* Biochemistry, 2001. **40**(14): p. 4211-21.
91. Powers, J.P., A. Rozek, and R.E. Hancock, *Structure-activity relationships for the beta-hairpin cationic antimicrobial peptide polyphemusin I.* Biochim Biophys Acta, 2004. **1698**(2): p. 239-50.

TH-2072_126106010

92. Bensch, K.W., et al., *hBD-1: a novel beta-defensin from human plasma.* FEBS Lett, 1995. **368**(2): p. 331-5.
93. Mygind, P.H., et al., *Plectasin is a peptide antibiotic with therapeutic potential from a saprophytic fungus.* Nature, 2005. **437**(7061): p. 975-80.
94. Fehlbaum, P., et al., *Insect immunity. Septic injury of Drosophila induces the synthesis of a potent antifungal peptide with sequence homology to plant antifungal peptides.* J Biol Chem, 1994. **269**(52): p. 33159-63.
95. Lamberty, M., et al., *Solution structures of the antifungal heliomicin and a selected variant with both antibacterial and antifungal activities.* Biochemistry, 2001. **40**(40): p. 11995-2003.
96. Selsted, M.E., et al., *Indolicidin, a novel bactericidal tridecapeptide amide from neutrophils.* J Biol Chem, 1992. **267**(7): p. 4292-5.
97. Rozek, A., C.L. Friedrich, and R.E. Hancock, *Structure of the bovine antimicrobial peptide indolicidin bound to dodecylphosphocholine and sodium dodecyl sulfate micelles.* Biochemistry, 2000. **39**(51): p. 15765-74.
98. Ebenhan, T., et al., *Antimicrobial peptides: their role as infection-selective tracers for molecular imaging.* Biomed Res Int, 2014. **2014**: p. 867381.
99. Schittek, B., et al., *Dermcidin: a novel human antibiotic peptide secreted by sweat glands.* Nat Immunol, 2001. **2**(12): p. 1133-7.
100. Lai, R., et al., *An anionic antimicrobial peptide from toad Bombina maxima.* Biochem Biophys Res Commun, 2002. **295**(4): p. 796-9.
101. Tossi, A. and L. Sandri, *Molecular diversity in gene-encoded, cationic antimicrobial polypeptides.* Curr Pharm Des, 2002. **8**(9): p. 743-61.
102. Wade, D. and J. Englund, *Synthetic antibiotic peptides database.* Protein Pept Lett, 2002. **9**(1): p. 53-7.
103. Whitmore, L. and B.A. Wallace, *The Peptaibol Database: a database for sequences and structures of naturally occurring peptaibols.* Nucleic Acids Res, 2004. **32**(Database issue): p. D593-4.
104. Leitgeb, B., et al., *The History of Alamethicin: A Review of the Most Extensively Studied Peptaibol.* Chemistry & Biodiversity, 2007. **4**(6): p. 1027-1051.
105. Brahmachary, M., et al., *ANTIMIC: a database of antimicrobial sequences.* Nucleic Acids Res, 2004. **32**(Database issue): p. D586-9.
106. Gueguen, Y., et al., *PenBase, the shrimp antimicrobial peptide penaeidin database: sequence-based classification and recommended nomenclature.* Dev Comp Immunol, 2006. **30**(3): p. 283-8.
107. Wang, C.K., et al., *CyBase: a database of cyclic protein sequences and structures, with applications in protein discovery and engineering.* Nucleic Acids Res, 2008. **36**(Database issue): p. D206-10.
108. Mulvenna, J.P., C. Wang, and D.J. Craik, *CyBase: a database of cyclic protein sequence and structure.* Nucleic Acids Res, 2006. **34**(Database issue): p. D192-4.
109. Hammami, R., et al., *BACTIBASE second release: a database and tool platform for bacteriocin characterization.* BMC Microbiol, 2010. **10**: p. 22.
110. Seebah, S., et al., *Defensins knowledgebase: a manually curated database and information source focused on the defensins family of antimicrobial peptides.* Nucleic Acids Res, 2007. **35**(Database issue): p. D265-8.
111. Fjell, C.D., R.E. Hancock, and A. Cherkasov, *AMPer: a database and an automated discovery tool for antimicrobial peptides.* Bioinformatics, 2007. **23**(9): p. 1148-55.
112. Li, Y. and Z. Chen, *RAPD: a database of recombinantly-produced antimicrobial peptides.* FEMS Microbiol Lett, 2008. **289**(2): p. 126-9.
113. Hammami, R., et al., *PhytAMP: a database dedicated to antimicrobial plant peptides.* Nucleic Acids Res, 2009. **37**(Database issue): p. D963-8.

TH-2072_126106010

114. Waghu, F.H., et al., *CAMPR3: a database on sequences, structures and signatures of antimicrobial peptides.* Nucleic Acids Res, 2016. **44**(D1): p. D1094-7.
115. Gogoladze, G., et al., *DBAASP: database of antimicrobial activity and structure of peptides.* FEMS Microbiol Lett, 2014. **357**(1): p. 63-8.
116. Schneewind, O. and D. Missiakas, *Lipoteichoic Acids, Phosphate-Containing Polymers in the Envelope of Gram-Positive Bacteria.* Journal of Bacteriology, 2014. **196**(6): p. 1133.
117. Percy, M.G. and A. Gründling, *Lipoteichoic Acid Synthesis and Function in Gram-Positive Bacteria.* Annual Review of Microbiology, 2014. **68**(1): p. 81-100.
118. Erridge, C., E. Bennett-Guerrero, and I.R. Poxton, *Structure and function of lipopolysaccharides.* Microbes and Infection, 2002. **4**(8): p. 837-851.
119. Matsuzaki, K., *Why and how are peptide-lipid interactions utilized for self-defense? Magainins and tachyplesins as archetypes.* Biochim Biophys Acta, 1999. **1462**(1-2): p. 1-10.
120. Powers, J.P. and R.E. Hancock, *The relationship between peptide structure and antibacterial activity.* Peptides, 2003. **24**(11): p. 1681-91.
121. Ehrenstein, G. and H. Lecar, *Electrically gated ionic channels in lipid bilayers.* Q Rev Biophys, 1977. **10**(1): p. 1-34.
122. Wu, M., et al., *Mechanism of interaction of different classes of cationic antimicrobial peptides with planar bilayers and with the cytoplasmic membrane of Escherichia coli.* Biochemistry, 1999. **38**(22): p. 7235-42.
123. Breukink, E. and B. de Kruijff, *The lantibiotic nisin, a special case or not?* Biochim Biophys Acta, 1999. **1462**(1-2): p. 223-34.
124. Qian, S., et al., *Structure of the alamethicin pore reconstructed by x-ray diffraction analysis.* Biophys J, 2008. **94**(9): p. 3512-22.
125. Hara, T., et al., *Effects of peptide dimerization on pore formation: Antiparallel disulfide-dimerized magainin 2 analogue.* Biopolymers, 2001. **58**(4): p. 437-46.
126. Yang, L., et al., *Crystallization of antimicrobial pores in membranes: magainin and protegrin.* Biophys J, 2000. **79**(4): p. 2002-9.
127. Ludtke, S.J., et al., *Membrane pores induced by magainin.* Biochemistry, 1996. **35**(43): p. 13723-8.
128. Matsuzaki, K., et al., *Mechanism of synergism between antimicrobial peptides magainin 2 and PGLa.* Biochemistry, 1998. **37**(43): p. 15144-53.
129. Sitaram, N. and R. Nagaraj, *Interaction of antimicrobial peptides with biological and model membranes: structural and charge requirements for activity.* Biochim Biophys Acta, 1999. **1462**(1-2): p. 29-54.
130. Lehrer, R.I., et al., *Interaction of human defensins with Escherichia coli. Mechanism of bactericidal activity.* J Clin Invest, 1989. **84**(2): p. 553-61.
131. Boman, H.G., B. Agerberth, and A. Boman, *Mechanisms of action on Escherichia coli of cecropin P1 and PR-39, two antibacterial peptides from pig intestine.* Infect Immun, 1993. **61**(7): p. 2978-84.
132. Cudic, M. and L. Otvos, Jr., *Intracellular targets of antibacterial peptides.* Curr Drug Targets, 2002. **3**(2): p. 101-6.
133. Kragol, G., et al., *The antibacterial peptide pyrrhocoricin inhibits the ATPase actions of DnaK and prevents chaperone-assisted protein folding.* Biochemistry, 2001. **40**(10): p. 3016-26.
134. Park, C.B., H.S. Kim, and S.C. Kim, *Mechanism of action of the antimicrobial peptide buforin II: buforin II kills microorganisms by penetrating the cell membrane and inhibiting cellular functions.* Biochem Biophys Res Commun, 1998. **244**(1): p. 253-7.
135. Yi, G.-S., et al., *Solution structure of an antimicrobial peptide buforin II.* FEBS Letters, 1996. **398**(1): p. 87-90.
136. del Castillo, F.J., I. del Castillo, and F. Moreno, *Construction and characterization of mutations at codon 751 of the Escherichia coli gyrB gene that confer resistance to the*

TH-2072_126106010

antimicrobial peptide microcin B17 and alter the activity of DNA gyrase. J Bacteriol, 2001. **183**(6): p. 2137-40.
137. Brotz, H., et al., *The lantibiotic mersacidin inhibits peptidoglycan synthesis by targeting lipid II.* Antimicrob Agents Chemother, 1998. **42**(1): p. 154-60.
138. Delcour, A.H., *Outer membrane permeability and antibiotic resistance.* Biochim Biophys Acta, 2009. **1794**(5): p. 808-16.
139. Malanovic, N. and K. Lohner, *Antimicrobial Peptides Targeting Gram-Positive Bacteria.* Pharmaceuticals (Basel), 2016. **9**(3).
140. Kosikowska, P. and A. Lesner, *Antimicrobial peptides (AMPs) as drug candidates: a patent review (2003-2015).* Expert Opin Ther Pat, 2016. **26**(6): p. 689-702.
141. Yang, D., et al., *Mammalian defensins in immunity: more than just microbicidal.* Trends Immunol, 2002. **23**(6): p. 291-6.
142. Bals, R. and J.M. Wilson, *Cathelicidins--a family of multifunctional antimicrobial peptides.* Cell Mol Life Sci, 2003. **60**(4): p. 711-20.
143. Bowdish, D.M., et al., *Impact of LL-37 on anti-infective immunity.* J Leukoc Biol, 2005. **77**(4): p. 451-9.
144. Brown, K.L. and R.E. Hancock, *Cationic host defense (antimicrobial) peptides.* Curr Opin Immunol, 2006. **18**(1): p. 24-30.
145. Yang, D., et al., *Human neutrophil defensins selectively chemoattract naive T and immature dendritic cells.* J Leukoc Biol, 2000. **68**(1): p. 9-14.
146. Hancock, R.E., K.L. Brown, and N. Mookherjee, *Host defence peptides from invertebrates--emerging antimicrobial strategies.* Immunobiology, 2006. **211**(4): p. 315-22.
147. Vallespi, M.G., et al., *A Limulus antilipopolysaccharide factor-derived peptide exhibits a new immunological activity with potential applicability in infectious diseases.* Clin Diagn Lab Immunol, 2000. **7**(4): p. 669-75.
148. Verhoef, J., *Antibiotic resistance: the pandemic.* Adv Exp Med Biol, 2003. **531**: p. 301-13.
149. Shea, K.M., *Antibiotic resistance: what is the impact of agricultural uses of antibiotics on children's health?* Pediatrics, 2003. **112**(1 Pt 2): p. 253-8.
150. Nizet, V., *Antimicrobial peptide resistance mechanisms of human bacterial pathogens.* Curr Issues Mol Biol, 2006. **8**(1): p. 11-26.
151. Andersson, D.I., D. Hughes, and J.Z. Kubicek-Sutherland, *Mechanisms and consequences of bacterial resistance to antimicrobial peptides.* Drug Resist Updat, 2016. **26**: p. 43-57.
152. Brown, S., J.P. Santa Maria, Jr., and S. Walker, *Wall teichoic acids of gram-positive bacteria.* Annu Rev Microbiol, 2013. **67**: p. 313-36.
153. Peschel, A., et al., *Inactivation of the dlt operon in Staphylococcus aureus confers sensitivity to defensins, protegrins, and other antimicrobial peptides.* J Biol Chem, 1999. **274**(13): p. 8405-10.
154. Abachin, E., et al., *Formation of D-alanyl-lipoteichoic acid is required for adhesion and virulence of Listeria monocytogenes.* Mol Microbiol, 2002. **43**(1): p. 1-14.
155. Ernst, C.M., et al., *The bacterial defensin resistance protein MprF consists of separable domains for lipid lysinylation and antimicrobial peptide repulsion.* PLoS Pathog, 2009. **5**(11): p. e1000660.
156. Samant, S., et al., *The Bacillus anthracis protein MprF is required for synthesis of lysylphosphatidylglycerols and for resistance to cationic antimicrobial peptides.* J Bacteriol, 2009. **191**(4): p. 1311-9.
157. McLeod, G.I. and M.P. Spector, *Starvation- and Stationary-phase-induced resistance to the antimicrobial peptide polymyxin B in Salmonella typhimurium is RpoS (sigma(S)) independent and occurs through both phoP-dependent and -independent pathways.* J Bacteriol, 1996. **178**(13): p. 3683-8.

TH-2072_126106010

158. Gunn, J.S., et al., *Genetic and functional analysis of a PmrA-PmrB-regulated locus necessary for lipopolysaccharide modification, antimicrobial peptide resistance, and oral virulence of Salmonella enterica serovar typhimurium.* Infect Immun, 2000. **68**(11): p. 6139-46.
159. Gooderham, W.J., et al., *The sensor kinase PhoQ mediates virulence in Pseudomonas aeruginosa.* Microbiology, 2009. **155**(Pt 3): p. 699-711.
160. Adams, M.D., et al., *Resistance to colistin in Acinetobacter baumannii associated with mutations in the PmrAB two-component system.* Antimicrob Agents Chemother, 2009. **53**(9): p. 3628-34.
161. Henderson, J.C., et al., *Antimicrobial peptide resistance of Vibrio cholerae results from an LPS modification pathway related to nonribosomal peptide synthetases.* ACS Chem Biol, 2014. **9**(10): p. 2382-92.
162. Friedrich, C., et al., *Salt-resistant alpha-helical cationic antimicrobial peptides.* Antimicrob Agents Chemother, 1999. **43**(7): p. 1542-8.
163. Guina, T., et al., *A PhoP-regulated outer membrane protease of Salmonella enterica serovar typhimurium promotes resistance to alpha-helical antimicrobial peptides.* J Bacteriol, 2000. **182**(14): p. 4077-86.
164. Stumpe, S., et al., *Identification of OmpT as the protease that hydrolyzes the antimicrobial peptide protamine before it enters growing cells of Escherichia coli.* J Bacteriol, 1998. **180**(15): p. 4002-6.
165. Ulvatne, H., et al., *Proteases in Escherichia coli and Staphylococcus aureus confer reduced susceptibility to lactoferricin B.* J Antimicrob Chemother, 2002. **50**(4): p. 461-7.
166. Ernst, R.K., T. Guina, and S.I. Miller, *How intracellular bacteria survive: surface modifications that promote resistance to host innate immune responses.* J Infect Dis, 1999. **179 Suppl 2**: p. S326-30.
167. Guo, L., et al., *Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides.* Cell, 1998. **95**(2): p. 189-98.
168. Lysenko, E.S., et al., *Bacterial phosphorylcholine decreases susceptibility to the antimicrobial peptide LL-37/hCAP18 expressed in the upper respiratory tract.* Infect Immun, 2000. **68**(3): p. 1664-71.
169. Kupferwasser, L.I., et al., *Plasmid-mediated resistance to thrombin-induced platelet microbicidal protein in staphylococci: role of the qacA locus.* Antimicrob Agents Chemother, 1999. **43**(10): p. 2395-9.
170. Liu, Y.Y., et al., *Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study.* Lancet Infect Dis, 2016. **16**(2): p. 161-8.
171. Mulvey, M.R., et al., *Dissemination of the mcr-1 colistin resistance gene.* Lancet Infect Dis, 2016. **16**(3): p. 289-90.
172. Gyurko, C., et al., *Candida albicans mutants deficient in respiration are resistant to the small cationic salivary antimicrobial peptide histatin 5.* Antimicrob Agents Chemother, 2000. **44**(2): p. 348-54.
173. Yin, L.M., et al., *Roles of hydrophobicity and charge distribution of cationic antimicrobial peptides in peptide-membrane interactions.* J Biol Chem, 2012. **287**(10): p. 7738-45.
174. Edwards, I.A., et al., *Contribution of Amphipathicity and Hydrophobicity to the Antimicrobial Activity and Cytotoxicity of beta-Hairpin Peptides.* ACS Infect Dis, 2016. **2**(6): p. 442-450.
175. Chen, Y., et al., *Role of peptide hydrophobicity in the mechanism of action of alpha-helical antimicrobial peptides.* Antimicrob Agents Chemother, 2007. **51**(4): p. 1398-406.
176. Evans, M.E., D.J. Feola, and R.P. Rapp, *Polymyxin B sulfate and colistin: old antibiotics for emerging multiresistant gram-negative bacteria.* Ann Pharmacother, 1999. **33**(9): p. 960-7.

TH-2072_126106010

177. McPhee, J.B., M.G. Scott, and R.E. Hancock, *Design of host defence peptides for antimicrobial and immunity enhancing activities.* Comb Chem High Throughput Screen, 2005. **8**(3): p. 257-72.
178. Olsen, J.V., S.E. Ong, and M. Mann, *Trypsin cleaves exclusively C-terminal to arginine and lysine residues.* Mol Cell Proteomics, 2004. **3**(6): p. 608-14.
179. Vlieghe, P., et al., *Synthetic therapeutic peptides: science and market.* Drug Discov Today, 2010. **15**(1-2): p. 40-56.
180. Hancock, R.E. and H.G. Sahl, *Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies.* Nat Biotechnol, 2006. **24**(12): p. 1551-7.
181. Porter, E.A., B. Weisblum, and S.H. Gellman, *Mimicry of host-defense peptides by unnatural oligomers: antimicrobial beta-peptides.* J Am Chem Soc, 2002. **124**(25): p. 7324-30.
182. Hamamoto, K., et al., *Antimicrobial activity and stability to proteolysis of small linear cationic peptides with D-amino acid substitutions.* Microbiol Immunol, 2002. **46**(11): p. 741-9.
183. Oh, J.E. and K.H. Lee, *Synthesis of novel unnatural amino acid as a building block and its incorporation into an antimicrobial peptide.* Bioorg Med Chem, 1999. **7**(12): p. 2985-90.
184. John, H., et al., *N-terminal acetylation protects glucagon-like peptide GLP-1-(7-34)-amide from DPP-IV-mediated degradation retaining cAMP- and insulin-releasing capacity.* Eur J Med Res, 2008. **13**(2): p. 73-8.
185. Khaksa, G., et al., *Pharmacokinetic study of niosome encapsulated insulin.* Indian J Exp Biol, 2000. **38**(9): p. 901-5.
186. Samad, A., Y. Sultana, and M. Aqil, *Liposomal drug delivery systems: an update review.* Curr Drug Deliv, 2007. **4**(4): p. 297-305.
187. Goldman, M.J., et al., *Human beta-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis.* Cell, 1997. **88**(4): p. 553-60.
188. Rothstein, D.M., et al., *Anticandida activity is retained in P-113, a 12-amino-acid fragment of histatin 5.* Antimicrob Agents Chemother, 2001. **45**(5): p. 1367-73.
189. Yu, H.Y., et al., *Easy strategy to increase salt resistance of antimicrobial peptides.* Antimicrob Agents Chemother, 2011. **55**(10): p. 4918-21.
190. Giuliani, A., G. Pirri, and S.F. Nicoletto, *Antimicrobial peptides: an overview of a promising class of therapeutics.* Central European Journal of Biology, 2007. **2**(1): p. 1-33.
191. Zavascki, A.P., et al., *Polymyxin B for the treatment of multidrug-resistant pathogens: a critical review.* J Antimicrob Chemother, 2007. **60**(6): p. 1206-15.
192. Gomes, A., C. Teixeira, and R. Ferraz, *Wound-Healing Peptides for Treatment of Chronic Diabetic Foot Ulcers and Other Infected Skin Injuries.* 2017. **22**(10).
193. Sader, H.S., et al., *Omiganan pentahydrochloride (MBI 226), a topical 12-amino-acid cationic peptide: spectrum of antimicrobial activity and measurements of bactericidal activity.* Antimicrob Agents Chemother, 2004. **48**(8): p. 3112-8.
194. Isaacson, R.E., *MBI-226. Micrologix/Fujisawa.* Curr Opin Investig Drugs, 2003. **4**(8): p. 999-1003.
195. Nilsson, A.C., et al., *LTX-109 is a novel agent for nasal decolonization of methicillin-resistant and -sensitive Staphylococcus aureus.* Antimicrob Agents Chemother, 2015. **59**(1): p. 145-51.
196. Velden, W.J., et al., *Safety and tolerability of the antimicrobial peptide human lactoferrin 1-11 (hLF1-11).* BMC Med, 2009. **7**: p. 44.
197. Shaykhiev, R., et al., *Human endogenous antibiotic LL-37 stimulates airway epithelial cell proliferation and wound closure.* Am J Physiol Lung Cell Mol Physiol, 2005. **289**(5): p. L842-8.
198. Gronberg, A., et al., *Treatment with LL-37 is safe and effective in enhancing healing of hard-to-heal venous leg ulcers: a randomized, placebo-controlled clinical trial.* Wound Repair Regen, 2014. **22**(5): p. 613-21.

TH-2072_126106010

199. Fox, J.L., *Antimicrobial peptides stage a comeback.* Nat Biotechnol, 2013. **31**(5): p. 379-82.
200. Nilsson, E., et al., *A novel polypeptide derived from human lactoferrin in sodium hyaluronate prevents postsurgical adhesion formation in the rat.* Ann Surg, 2009. **250**(6): p. 1021-8.
201. Wiig, M.E., et al., *PXL01 in sodium hyaluronate for improvement of hand recovery after flexor tendon repair surgery: randomized controlled trial.* PLoS One, 2014. **9**(10): p. e110735.
202. Edsfeldt, S., et al., *PXL01 in sodium hyaluronate results in increased PRG4 expression: a potential mechanism for anti-adhesion.* Ups J Med Sci, 2017. **122**(1): p. 28-34.
203. Elad, S., et al., *The antimicrobial effect of Iseganan HCl oral solution in patients receiving stomatotoxic chemotherapy: analysis from a multicenter, double-blind, placebo-controlled, randomized, phase III clinical trial.* J Oral Pathol Med, 2012. **41**(3): p. 229-34.
204. Ling, L.L., et al., *A new antibiotic kills pathogens without detectable resistance.* Nature, 2015. **517**(7535): p. 455-9.
205. Lv, Y., et al., *Antimicrobial properties and membrane-active mechanism of a potential alpha-helical antimicrobial derived from cathelicidin PMAP-36.* PLoS One, 2014. **9**(1): p. e86364.
206. Raghuraman, H. and A. Chattopadhyay, *Influence of lipid chain unsaturation on membrane-bound melittin: a fluorescence approach.* Biochim Biophys Acta, 2004. **1665**(1-2): p. 29-39.
207. Greenfield, N.J., *Using circular dichroism spectra to estimate protein secondary structure.* Nat Protoc, 2006. **1**(6): p. 2876-90.
208. Savitzky, A. and M.J.E. Golay, *Smoothing and Differentiation of Data by Simplified Least Squares Procedures.* Analytical Chemistry, 1964. **36**(8): p. 1627-1639.
209. Pain, R., *Determining the CD spectrum of a protein.* Curr Protoc Protein Sci, 2005. **Chapter 7**: p. Unit 7.6.
210. Wiegand, I., K. Hilpert, and R.E. Hancock, *Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances.* Nat Protoc, 2008. **3**(2): p. 163-75.
211. Salje, J., et al., *Direct membrane binding by bacterial actin MreB.* Mol Cell, 2011. **43**(3): p. 478-87.
212. Bi, X., et al., *Investigation of the role of tryptophan residues in cationic antimicrobial peptides to determine the mechanism of antimicrobial action.* J Appl Microbiol, 2013. **115**(3): p. 663-72.
213. Pasupuleti, M., et al., *End-tagging of ultra-short antimicrobial peptides by W/F stretches to facilitate bacterial killing.* PLoS One, 2009. **4**(4): p. e5285.
214. Pasupuleti, M., et al., *Tryptophan end-tagging of antimicrobial peptides for increased potency against Pseudomonas aeruginosa.* Biochim Biophys Acta, 2009. **1790**(8): p. 800-8.
215. Gautier, R., et al., *HELIQUEST: a web server to screen sequences with specific alpha-helical properties.* Bioinformatics, 2008. **24**(18): p. 2101-2.
216. Zhou, C., et al., *High potency and broad-spectrum antimicrobial peptides synthesized via ring-opening polymerization of alpha-aminoacid-N-carboxyanhydrides.* Biomacromolecules, 2010. **11**(1): p. 60-7.
217. Andreu, D., et al., *Solid-phase synthesis of cecropin A and related peptides.* Proc Natl Acad Sci U S A, 1983. **80**(21): p. 6475-9.
218. Torcato, I.M., et al., *Design and characterization of novel antimicrobial peptides, R-BP100 and RW-BP100, with activity against Gram-negative and Gram-positive bacteria.* Biochim Biophys Acta, 2013. **1828**(3): p. 944-55.
219. Lum, K.Y., et al., *Activity of Novel Synthetic Peptides against Candida albicans.* Sci Rep, 2015. **5**: p. 9657.

TH-2072_126106010

220. Herricks, J.R., D. Nguyen, and W. Margolin, *A thermosensitive defect in the ATP binding pocket of FtsA can be suppressed by allosteric changes in the dimer interface.* Mol Microbiol, 2014. **94**(3): p. 713-27.
221. Pichoff, S. and J. Lutkenhaus, *Tethering the Z ring to the membrane through a conserved membrane targeting sequence in FtsA.* Mol Microbiol, 2005. **55**(6): p. 1722-34.
222. Pichoff, S., et al., *FtsA mutants impaired for self-interaction bypass ZipA suggesting a model in which FtsA's self-interaction competes with its ability to recruit downstream division proteins.* Mol Microbiol, 2012. **83**(1): p. 151-67.
223. Woody, R.W., *Theory of Circular Dichroism of Proteins*, in *Circular Dichroism and the Conformational Analysis of Biomolecules*, G.D. Fasman, Editor. 1996, Springer US: Boston, MA. p. 25-67.
224. Perczel, A. and M. Hollosi, *Turns*, in *Circular Dichroism and the Conformational Analysis of Biomolecules*, G.D. Fasman, Editor. 1996, Springer: Boston, MA. p. 285-380.
225. Woody, R.W. and A.K. Dunker, *Aromatic and Cystine Side-Chain Circular Dichroism in Proteins*, in *Circular Dichroism and the Conformational Analysis of Biomolecules*, G.D. Fasman, Editor. 1996, Springer US: Boston, MA. p. 109-157.
226. Friedrich, C.L., et al., *Antibacterial action of structurally diverse cationic peptides on gram-positive bacteria.* Antimicrob Agents Chemother, 2000. **44**(8): p. 2086-92.
227. Hassan, M., et al., *Natural antimicrobial peptides from bacteria: characteristics and potential applications to fight against antibiotic resistance.* J Appl Microbiol, 2012. **113**(4): p. 723-36.
228. Eftink, M.R. and C.A. Ghiron, *Fluorescence quenching of indole and model micelle systems.* The Journal of Physical Chemistry, 1976. **80**(5): p. 486-493.
229. McManus, A.M., et al., *Three-Dimensional Structure of RK-1: A Novel α-Defensin Peptide.* Biochemistry, 2000. **39**(51): p. 15757-15764.
230. Hristova, K., M.E. Selsted, and S.H. White, *Critical Role of Lipid Composition in Membrane Permeabilization by Rabbit Neutrophil Defensins.* Journal of Biological Chemistry, 1997. **272**(39): p. 24224-24233.
231. Craik, D.J., et al., *The future of peptide-based drugs.* Chem Biol Drug Des, 2013. **81**(1): p. 136-47.
232. Tavares, L.S., et al., *Strategies and molecular tools to fight antimicrobial resistance: resistome, transcriptome, and antimicrobial peptides.* Front Microbiol, 2013. **4**: p. 412.
233. Malanovic, N. and K. Lohner, *Antimicrobial Peptides Targeting Gram-Positive Bacteria.* Pharmaceuticals, 2016. **9**(3).
234. Goldman, M.J., et al., *Human β-Defensin-1 Is a Salt-Sensitive Antibiotic in Lung That Is Inactivated in Cystic Fibrosis.* Cell, 1997. **88**(4): p. 553-560.
235. Lee, J.Y., et al., *Salt-resistant homodimeric bactenecin, a cathelicidin-derived antimicrobial peptide.* The FEBS Journal, 2008. **275**(15): p. 3911-3920.
236. Varkey, J. and R. Nagaraj, *Antibacterial activity of human neutrophil defensin HNP-1 analogs without cysteines.* Antimicrob Agents Chemother, 2005. **49**(11): p. 4561-6.
237. Varkey, J., S. Singh, and R. Nagaraj, *Antibacterial activity of linear peptides spanning the carboxy-terminal beta-sheet domain of arthropod defensins.* Peptides, 2006. **27**(11): p. 2614-23.
238. Waterer, G.W. and R.G. Wunderink, *Increasing threat of Gram-negative bacteria.* Crit Care Med, 2001. **29**(4 Suppl): p. N75-81.
239. Daxboeck, F., et al., *Economic burden associated with multi-resistant Gram-negative organisms compared with that for methicillin-resistant Staphylococcus aureus in a university teaching hospital.* J Hosp Infect, 2006. **62**(2): p. 214-8.
240. Rossolini, G.M., et al., *Update on the antibiotic resistance crisis.* Curr Opin Pharmacol, 2014. **18**: p. 56-60.

TH-2072_126106010

241. Hover, B.M., et al., *Culture-independent discovery of the malacidins as calcium-dependent antibiotics with activity against multidrug-resistant Gram-positive pathogens.* 2018. **3**(4): p. 415-422.
242. Ventola, C.L., *The antibiotic resistance crisis: part 1: causes and threats.* P t, 2015. **40**(4): p. 277-83.
243. Lister, P.D., D.J. Wolter, and N.D. Hanson, *Antibacterial-resistant Pseudomonas aeruginosa: clinical impact and complex regulation of chromosomally encoded resistance mechanisms.* Clin Microbiol Rev, 2009. **22**(4): p. 582-610.
244. Chambers, H.F. and F.R. Deleo, *Waves of resistance: Staphylococcus aureus in the antibiotic era.* Nat Rev Microbiol, 2009. **7**(9): p. 629-41.
245. Enright, M.C., et al., *The evolutionary history of methicillin-resistant Staphylococcus aureus (MRSA).* Proc Natl Acad Sci U S A, 2002. **99**(11): p. 7687-92.
246. Padhi, A., et al., *Antimicrobial peptides and proteins in mycobacterial therapy: current status and future prospects.* Tuberculosis (Edinb), 2014. **94**(4): p. 363-73.
247. Gutsmann, T., *Interaction between antimicrobial peptides and mycobacteria.* Biochim Biophys Acta, 2016. **1858**(5): p. 1034-43.
248. Raghavendra, T., S. Patil, and R. Mukherjee, *Peptidoglycan in Mycobacteria: chemistry, biology and intervention.* Glycoconjugate Journal, 2018. **35**(5): p. 421-432.
249. Jarlier, V. and H. Nikaido, *Permeability barrier to hydrophilic solutes in Mycobacterium chelonei.* Journal of Bacteriology, 1990. **172**(3): p. 1418.

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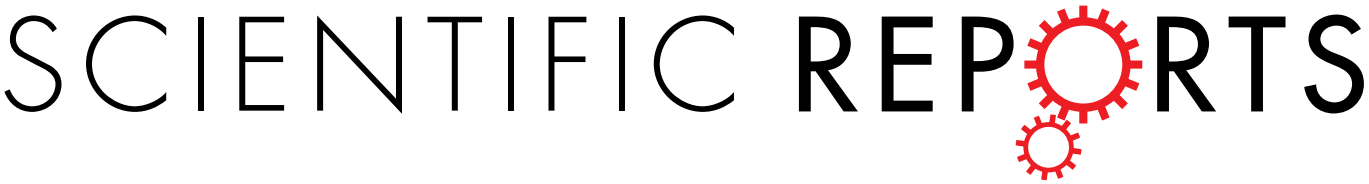

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Highly potent antimicrobial peptides from N-terminal membrane-binding region of *E. coli* MreB

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Karabi Saikia, Yalavarthi Durga Sravani, Vibin Ramakrishnan & Nitin Chaudhary

Microbial pathogenesis is a serious health concern. The threat escalates as the existing conventional antimicrobials are losing their efficacy against the evolving pathogens. Peptides hold promise to be developed into next-generation antibiotics. Antimicrobial peptides adopt amphipathic structures that could selectively bind to and disrupt the microbial membranes. Interaction of proteins with membranes is central to all living systems and we reasoned that the membrane-binding domains in microbial proteins could be developed into efficient antimicrobials. This is an interesting approach as self-like sequences could elude the microbial strategies of degrading the antimicrobial peptides, one of the mechanisms of showing resistance to antimicrobials. We selected the 9-residue-long membrane-binding region of *E. coli* MreB protein. The 9-residue peptide (C-terminal amide) and its N-terminal acetylated analog displayed broad-spectrum activity, killing Gram-negative bacteria, Gram-positive bacteria, and fungi. Extension with a tryptophan residue at the N-terminus drastically improved the activity of the peptides with lethal concentrations $\leq$10 μM against all the organisms tested. The tryptophan-extended peptides caused complete killing of *C. albicans* as well as gentamicin and methicillin resistant *S. aureus* at 5 μM concentration. Lipid-binding studies and electron microscopic analyses of the peptide-treated microbes suggest membrane disruption as the mechanism of killing.

Abuse of antibiotics has led to an alarming situation wherein many of the clinical strains have developed resistance against multiple antibiotics available. Emerging resistance to the existing antibiotics together with very slow discovery of new class of antibiotics is one of the major health threats today. Higher organisms have cohabited with microorganisms on this planet throughout their evolution and have devised strategies to control and combat them. Antimicrobial peptides (AMPs) happen to be one of the key members of such defense arsenal and play an important role to ward off the pathogens[1]. AMPs occur naturally in almost all organisms as an important element of their innate immune system[2]. AMPs have been exposed to microbes for millions of years but resistance against them is not prevalent. Moreover, during the course of evolution, mutations in the microbes have led to the diversification of AMPs[1]. The sequence diversity of the AMPs is such that classifying them based on their sequences is neither practical nor useful. Such diversity in AMPs could account for the inability of the microbes to develop good resistance against them thereby helping such an ancient weapon flourish throughout evolution. The activity of peptides could be altered by making subtle changes in their amino acid sequence, composition, and conformation thereby suggesting that the AMPs hold the promise to be developed into the next-generation antibiotics[3].

Cationic antimicrobial peptides (CAMPs) i.e. the peptides possessing a net positive charge at neutral pH, constitute the largest group of the antimicrobial peptides[4]. Structurally, these peptides could attain diverse conformations such as α-helices, β-sheets, mixed conformations, loops, and extended structures[5]. An important feature of AMPs is that they fold into amphipathic structures that could interact with bacterial membranes[6]. Amphipathic α-helical peptides constitute the largest structural class among all the known CAMPs[7]. It is imperative to design antimicrobials that are cost effective and possess broad spectrum antimicrobial activity without instigating any toxic effect to the host. Naturally occurring antimicrobial peptides are often 20–50 residues long and high cost of peptide synthesis is one of the major obstacles in developing peptides as antibiotics[8,9]. The focus, therefore, is shifting towards designing and developing shorter antimicrobial peptides[10].

Department of Biosciences and Bioengineering Indian Institute of Technology Guwahati, Guwahati – 781 039, India. Correspondence and requests for materials should be addressed to N.C. (email: chaudhary@iitg.ernet.in)

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Peptide name	Peptide sequence[a]	z[b]	⟨H⟩[c]	⟨μH⟩[c]	D
$MreB_{1-9}$	MLKKFRGMF-am	+4	0.528	0.762	2.039
Ac-$MreB_{1-9}$	Ac-MLKKFRGMF-am	+3	0.528	0.762	1.709
W-$MreB_{1-9}$	WMLKKFRGMF-am	+4	0.7	0.523	1.814
Ac-W-$MreB_{1-9}$	Ac-WMLKKFRGMF-am	+3	0.7	0.523	1.484

Table 1. Sequences and physicochemical properties of the peptides. [a]'Ac-' at N-terminus represents acetylated amino-terminus, while '-am' at C-terminus represents C-terminus amide. [b]Net charge on the peptide at pH 7.4 [c]⟨H⟩ and ⟨μH⟩ were calculated using Heliquest web server[12].

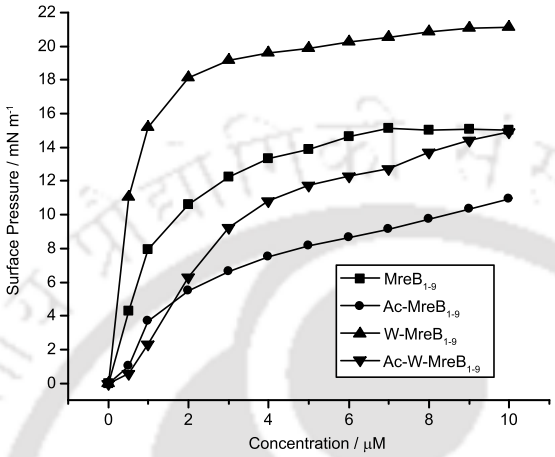

Figure 1. Surface activity of the peptides. Peptides at concentrations ranging from 0.5–10 μM were injected into the subphase. The stabilized surface pressure values are plotted against the peptide concentration.

MreB is a bacterial cytoskeleton protein present in non-spherical cells localized beneath the cell membrane in the form of filaments[11]. MreB attributes to the survival of non-spherical bacteria by maintaining the cellular structure akin to the cytoskeletal elements like actin, tubulin, and intermediate filaments present in eukaryotic cells. MreB proteins exist in two different forms, one with a short (~7–9) amino acid stretch at N-terminus that could fold into an amphipathic α-helix and the other that lacks this N-terminal region[11]. The N-terminal amphipathic helix in *E. coli* MreB is both necessary and sufficient for its membrane binding. We predicted that this N-terminal stretch could be developed into a potent antimicrobial peptide. Development of an amino acid sequence of microbial origin into an antimicrobial peptide is an interesting strategy as microbes should find it difficult to counter self-like sequences through conventional membrane modifications that are effected by resistant bacteria. Furthermore, the peptide could interfere with the native peptide stretches thereby disturbing the natural microbial processes.

Results

Peptides. The 9-residue peptide stretch (MLKKFRGMF) from *E. coli* was selected and four peptides were designed (Table 1).

Mean hydrophobicity (⟨H⟩) and mean hydrophobic helical moments (⟨μH⟩) were calculated using Heliquest web server[12]. A discrimination factor (D) is defined as 0.944 ⟨μH⟩ +0.33(z) to identify the possible lipid-binding helices[12,13]. If the discrimination factor is higher than 1.34, the helix is predicted to be a membrane-binding one. As per the Heliquest discrimination factor criteria, all the four peptides qualified to be the lipid-binding ones. The peptides were synthesized, purified, and their identities ascertained using MALDI mass spectrometry (see Supplementary Figs S1 and S2).

Surface activity and membrane binding of the peptides. Surface activity of the peptides was measured using 10 mM phosphate buffer, pH 7.4 as the aqueous subphase. Peptides were injected into the subphase and changes in the surface pressure were monitored by the Wilhelmy method using a platinum plate. All the peptides caused substantial enhancement in the surface pressure ($\geq$5 mN m^{-1}) even at 2 μM peptide concentration (Fig. 1).

The change in surface pressure, by and large, is higher for the peptides with higher net charge. Among the peptides with identical net charge, the longer peptides displayed larger changes in the surface pressure. Interaction of peptides with lipids was studied using lipid monolayers. 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) were used as the zwitterionic and negatively-charged lipids, respectively. Lipid monolayers of POPC and 7:3 POPC:POPG were prepared so as to obtain the surface pressures ~30–32 mN m^{-1}. Without disturbing the lipid monolayers, the peptides were injected into the subphase so as to have 10 μM concentration. The peptides caused ~2–6 mN m^{-1} increase in the surface pressure for the POPC monolayer (Table 2). Ac-W-$MreB_{1-9}$ caused 5.8 mN m^{-1} increase in the surface pressure

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Lipid monolayer	Increase in surface pressure (mN m^{-1})			
	$MreB_{1-9}$	Ac-$MreB_{1-9}$	W-$MreB_{1-9}$	Ac-W-$MreB_{1-9}$
POPC	3.6	1.8	3.6	5.8
POPC:POPG (7:3)	8.3	8.5	8.7	10

Table 2. Maximum increase obtained in the surface pressure of the lipid monolayers on peptide addition (10 μM initial subphase concentration).

Microbe	Minimum Lethal Concentration (μM)[a]			
	$MreB_{1-9}$	Ac-$MreB_{1-9}$	W-$MreB_{1-9}$	Ac-W-$MreB_{1-9}$
Escherichia coli	20	15	5	10
Pseudomonas aeruginosa	10	100	3	10
Salmonella enterica	10	15	3	2
Staphylococcus aureus	50	100	10	10
Staphylococcus aureus (*Gentamicin and Methicillin-resistant*)	20	20	5	5
Candida albicans	15	15	5	5

Table 3. Antimicrobial activity of the peptides. The assays were carried out with the mid-log phase cells in 10 mM phosphate buffer, pH 7.4. [a]Minimum peptide concentration that resulted in complete killing of the bacteria/fungus.

while $\leq$3.6 mN m^{-1} change was observed for the other three peptides. With POPC:POPG monolayers, all four peptides caused large enhancement in the surface pressure. Ac-W-$MreB_{1-9}$ caused 10 mN m^{-1} increase in surface pressure compared to 5.8 mN m^{-1} increase caused to POPC monolayers. Other three peptides caused ~8.5 mN m^{-1} increase to POPC:POPG surface pressure compared to $\leq$3.6 mN m^{-1} increase caused to POPC monolayers.

As predicted, all the peptides turn out to be the membrane-binding ones. The data clearly suggest that the peptides preferentially bind to the negatively-charged lipids as expected from the cationic, amphipathic peptides.

Antimicrobial activity. Antimicrobial activity of the peptides was investigated against Gram-positive and Gram-negative bacteria as well as fungus. The minimal lethal concentrations of the peptides are shown in Table 3.

$MreB_{1-9}$, the native sequence, possesses a net charge of +4 and exhibit high antimicrobial activity against both Gram-positive and Gram-negative bacteria as well as *C. albicans*. All the microorganisms tested, other than *S. aureus*, could be efficiently killed at 20 μM or lesser peptide concentration. The lethal concentration for *S. aureus* was 50 μM. Gentamicin-methicillin-resistant *S. aureus* (gentamicin-resistant MRSA), however, appeared more susceptible (lethal $MreB_{1-9}$ concentration = 20 μM). Capping of N-terminus with acetyl group renders the peptide less effective, particularly against *P. aeruginosa* and *S. aureus*. Interestingly, the activity against *C. albicans* and gentamicin-resistant MRSA is not affected on N-terminal acetylation. Extension of $MreB_{1-9}$ by a tryptophan residue at N-terminus makes the peptide (W-$MreB_{1-9}$) at least 3 times more active. All the microbes tested were efficiently killed at $\leq$10 μM peptide concentration. Bacterial membranes are negatively charged and most cationic amphipathic antimicrobial peptides kill the bacteria by disrupting their membranes. A decrease in cationicity would adversely affect the peptide binding to microbial membranes thereby compromising their activity.

The kinetics of killing in the presence of the lethal concentration of the peptides was examined (Fig. 2). The peptides exhibited strain-dependent killing-kinetics. All the four peptides caused rapid killing of *S. enterica*; the bacteria was completely killed within 15 minutes of peptide treatment. *S. aureus*, on the other hand, showed >50% survival even 1 h after the peptide treatment. The bacteria, however, was completely killed in 2 hours. Ac-W-$MreB_{1-9}$ displayed the most rapid killing against *E. coli*, *P. aeruginosa*, *S. enterica*, and *C. albicans*. All the three bacteria were completely killed within 5 minutes of peptide treatment while *C. albicans* was killed within 15 minutes. Killing of *S. aureus* was slower and all the peptides followed very similar kinetics. The kinetics of killing are comparable or better than several established antimicrobial peptides[14,15].

Field emission scanning electron microscopic (FESEM) images of the peptide-treated microbes are shown in Fig. 3. The peptide-treated microbes displayed unusual morphology. Treatment with the peptides caused pore formation in the bacterial membrane alongside large-scale perturbation. Similar membrane perturbation was observed for *C. albicans* as well. The microscopic analysis suggests membrane perturbation as the mechanism of killing.

Salt sensitivity. The peptides retained substantial activity in the presence of 100 mM NaCl (Table 4). All the peptides displayed >75% killing of all the bacteria at their lethal concentrations. The activity against *C. albicans*, however, reduced to 38–54%.

Antibacterial activity of Ac-W-$MreB_{1-9}$ was little affected by the presence of salt showing complete killing of *E. coli*, *P. aeruginosa*, and *S. enterica;* and >90% killing of *S. aureus*. Activity against *C. albicans*, however reduced to ~43%. Divalent cations showed varied affects on the activity of the peptides. $MreB_{1-9}$ retained >50% activity against all the microorganisms tested. Activity of Ac-$MreB_{1-9}$, on the other hand, was severely compromised against *E. coli* and *S. enterica* in the presence of divalent cations. Both $MreB_{1-9}$ and Ac-$MreB_{1-9}$ retained >90%

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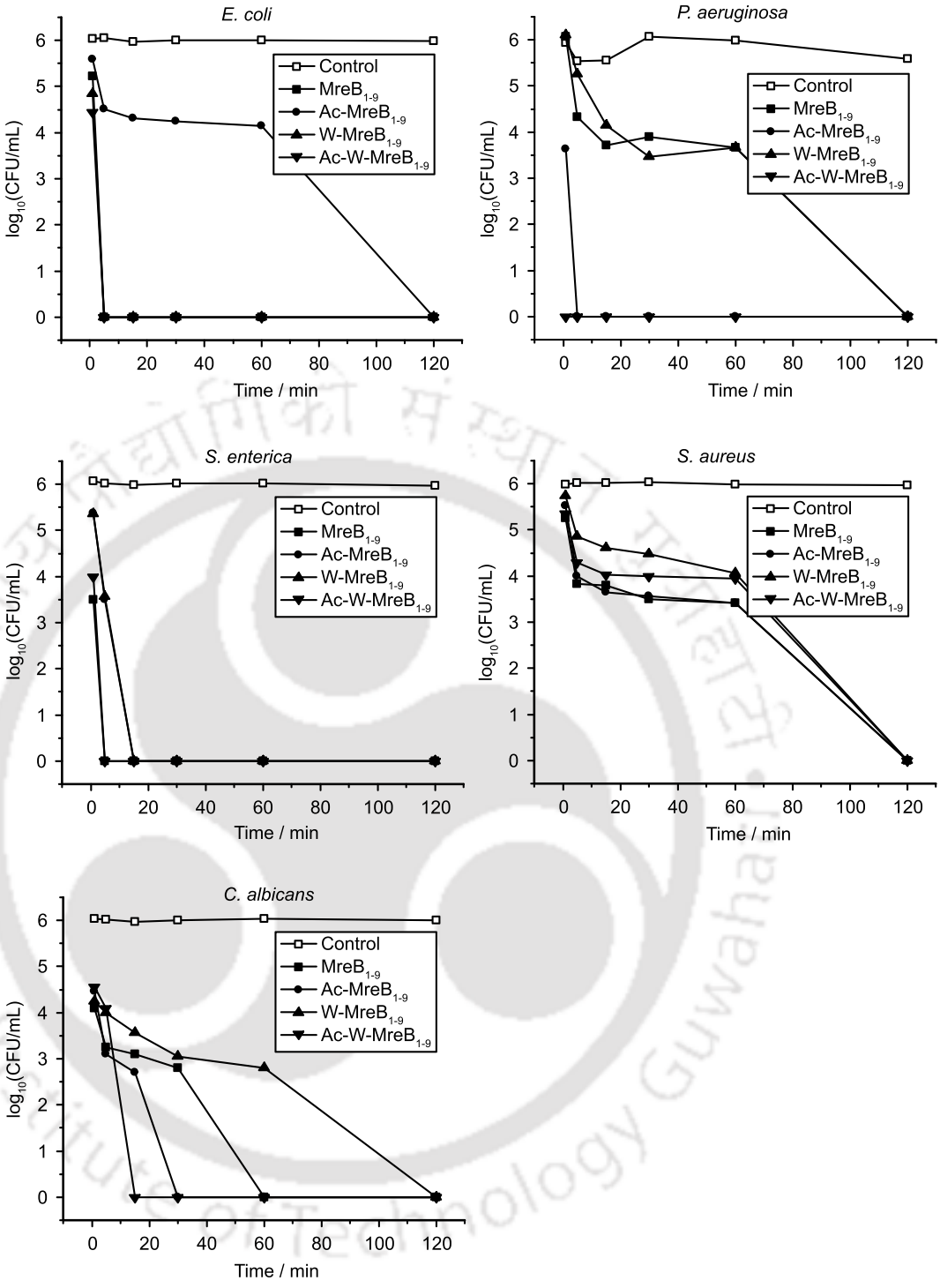

Figure 2. Kinetics of microbial killing by MreB-derived peptides. Microbes were treated with the peptides at their minimum lethal concentrations. Aliquots were taken out at different time points and antimicrobial activity was assayed as described in the 'Materials and methods' section.

activity against *S. aureus* in the presence of salt as well as divalent cations. Tryptophan-extended peptides exhibited better activity than the native sequences. The activity of W-MreB$_{1-9}$ against *E. coli* and *P. aeruginosa*, however, got severely compromised in the presence of Mg^{2+} ions. Ac-W-MreB$_{1-9}$, on the other hand, retained ~80% or more activity against all the four bacteria as well as fungus in the presence of divalent cations. MreB$_{1-9}$ is the sequence derived from *E. coli* and it is interesting to see that the native MreB peptide, MreB$_{1-9}$ retains >50% activity against *E. coli* in the presence of salt and divalent cations. The varied response to the divalent cations suggests that the peptides could work synergistically to achieve broad spectrum killing under physiological conditions.

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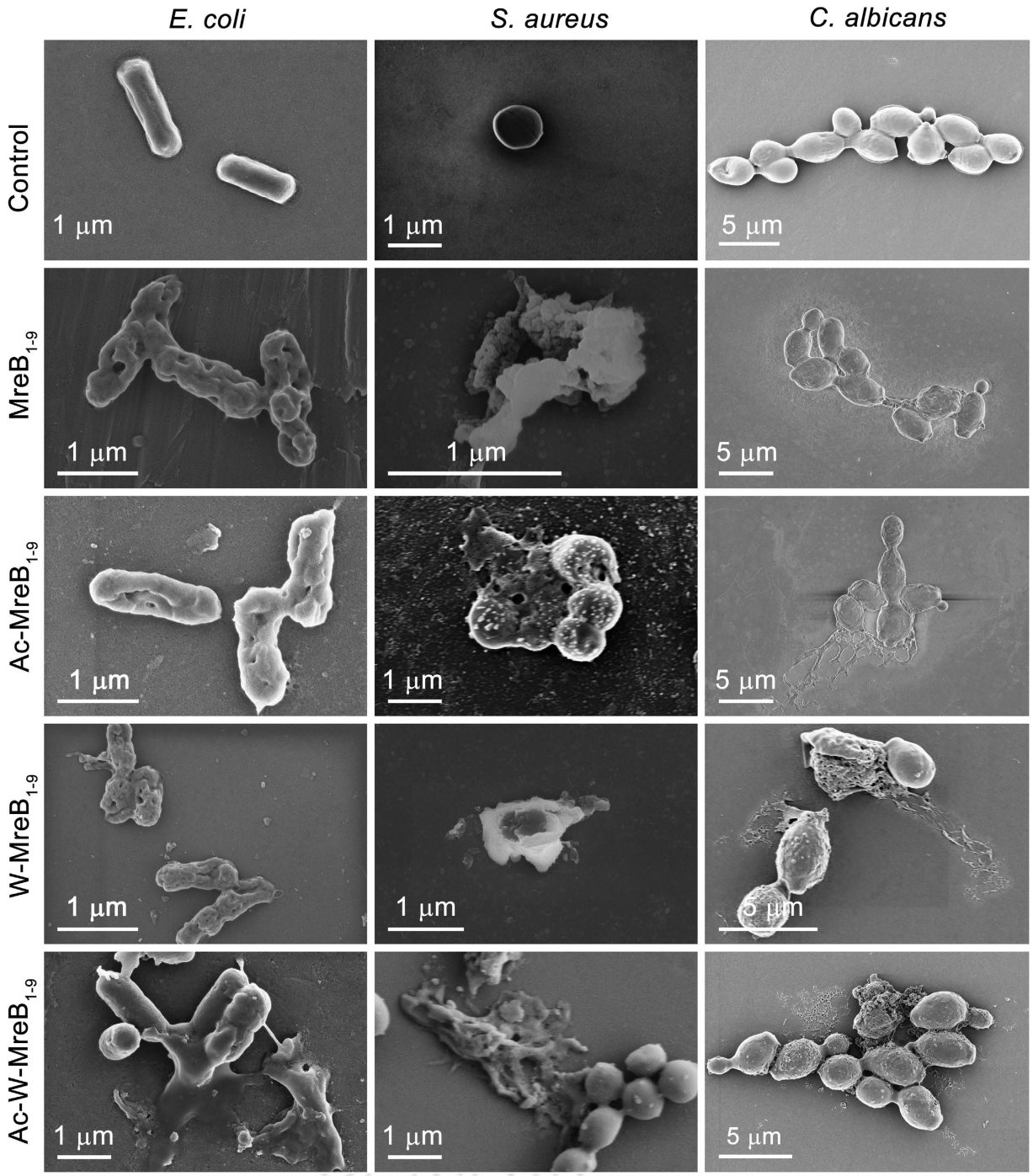

Figure 3. FESEM images of the peptide-treated *E. coli*, *S. aureus*, and *C. albicans*. Microbes were treated with the peptides at their minimum lethal concentrations and incubated at 37 °C (bacteria) and 28 °C (fungus) for 2 hours. The cells were harvested, fixed, and analysed using FESEM as described in the 'Materials and methods' section.

Hemolytic assay. Lysis of human erythrocytes by the peptides was examined at 50 and 100 μM peptide concentrations (Table 5). No hemolysis was caused by $MreB_{1-9}$, Ac-$MreB_{1-9}$, and W-$MreB_{1-9}$ at 50 μM concentration while ~4% hemolysis was caused by Ac-W-$MreB_{1-9}$. The lethal concentrations of W-$MreB_{1-9}$ and Ac-W-$MreB_{1-9}$ are ≤10 μM against all the organisms tested (Table 3). W-$MreB_{1-9}$ did not cause any hemolysis while Ac-W-$MreB_{1-9}$ caused <5% hemolysis at 5-fold higher concentrations. W-$MreB_{1-9}$ caused little hemolysis (1.62%) even at 10-fold higher concentration.

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	Microbe → Peptide ↓		*E. coli*	*P. aeruginosa*	*S. enterica*	*S. aureus*	*Gentamicin-resistant MRSA*	*C. albicans*
Percentage killing	$MreB_{1-9}$	NaCl	99.7 ± 0.4	90.0 ± 13.9	96.6 ± 2.7	98.9 ± 1.3	96.7 ± 2.7	38.1 ± 5.9
		$MgCl_2$	53 ± 9.6	76.2 ± 8.1	79.1 ± 1.0	90.8 ± 9.2	98.8 ± 0.7	98.0 ± 1.6
		$CaCl_2$	69.5 ± 1.4	99.7 ± 0.4	52.4 ± 10.8	98.0 ± 2.1	95.6 ± 4.1	99.6 ± 0.2
	Ac-$MreB_{1-9}$	NaCl	98.6 ± 1.9	97.8 ± 1.4	78.6 ± 11.0	99.6 ± 0.6	93.2 ± 7.7	54.0 ± 9.6
		$MgCl_2$	0	42.0 ± 5.8	65.6 ± 6.7	93.6 ± 9.2	97.2 ± 3.6	98.5 ± 1.0
		$CaCl_2$	4.6 ± 3.4	100	2.5 ± 1.1	97.4 ± 3.9	96.4 ± 1.8	57.0 ± 6.1
	W-$MreB_{1-9}$	NaCl	99.7 ± 0.4	93.0 ± 9.7	98.2 ± 0.1	87.6 ± 3.0	96.8 ± 3.5	45.7 ± 8.8
		$MgCl_2$	0	15.1 ± 4.1	72.7 ± 9.4	91.7 ± 7.8	97.9 ± 2.7	96.8 ± 3.2
		$CaCl_2$	10.4 ± 3.2	99.8 ± 0.1	99.5 ± 0.2	74.5 ± 2.9	95.8 ± 4.8	58.3 ± 12.0
	Ac-W-$MreB_{1-9}$	NaCl	100	100	100	93.5 ± 9.7	92.0 ± 8.8	43.4 ± 8.9
		$MgCl_2$	100	100	96.7 ± 3.8	79.9 ± 4.9	95.7 ± 3.0	99.9 ± 0.1
		$CaCl_2$	100	100	100	83.5 ± 1.4	89.6 ± 3.3	92.5 ± 8.8

Table 4. Antimicrobial activity of the MreB-derived peptides at their lethal concentrations in the presence of 100 mM NaCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$.

Peptide concentration (μM)	Percentage hemolysis			
	$MreB_{1-9}$	Ac- $MreB_{1-9}$	W- $MreB_{1-9}$	Ac-W- $MreB_{1-9}$
50	0	0	0	4.15 ± 0.96
100	0	0	1.62 ± 0.08	11.88 ± 2.44

Table 5. Percentage lysis of human erythrocytes by the MreB-derived peptides.

Discussion

Emergence of multidrug-resistant microbes demands for the development of new class of antimicrobials and peptides are being looked at with high expectations[16]. Antimicrobial peptides are natural defense molecules found in all life forms. They have sustained the evolutionary pressure without causing any significant antimicrobial resistance making them the molecules of choice for developing the new generation of antimicrobials.

We realized the antimicrobial potential of the 9-residue long N-terminal amphipathic helix of *E. coli* MreB protein and designed four peptides. The peptides displayed broad spectrum activity, killing Gram-negative bacteria (*E. coli*, *P. aeruginosa*, *S. enterica*), Gram-positive bacteria (*S. aureus* and gentamicin-resistant MRSA), and fungus (*C. albicans*). The lack of discovery of the new antibiotics against Gram-negative bacteria is alarming. The new antibiotics that entered medicine in last five decades were all against the Gram-positive bacteria. Largely due to the lack of new antibiotics that could combat Gram-negative bacteria, Gram-negative bacteremia has become a serious health issue[9,17,18]. Among Gram-negative bacteria, *P. aeruginosa* turns out to be one of the most common human pathogens. It is one of the two most common pathogens causing nosocomial pneumonia, *S. aureus* being the other one. *P. aeruginosa* is an opportunistic pathogen and the serious Pseudomonal infections are often hospital acquired[19]. It happens to be the most commonly isolated pathogen from patients that are hospitalized for long durations especially the ones that have compromised immune system. *P. aeruginosa* is life-threatening for the critically ill patients in the intensive care units. It is an intrinsically resistant bacterium to many antibiotics and is capable of acquiring resistance to multiple antibiotics[17,19]. *E. coli* and *S. enterica* are other clinically-relevant Gram-negative bacteria. All three bacteria were killed by the native sequence, $MreB_{1-9}$ at lethal concentrations comparable to many established antimicrobial peptides[20]. Extension of $MreB_{1-9}$ at the N-terminus by a tryptophan residue improved the activities drastically. All three Gram-negative bacteria could be completely killed at 5 μM or lower peptide concentration, an activity comparable or better than many highly-active antimicrobial peptides[20]. Indolicidin, for example, displays an MIC of 67 μM against *P. aeruginosa*, *S. aureus*, and *C. albicans*; the MIC against *E. coli* was >134 μM. Human cathelicidin, LL-37 displays an MIC >50 μM against *E. coli*, *P. aeruginosa*, *S. aureus*, and *C. albicans*. Melittin, the highly lytic peptide from honey bee venom displays an MIC of 2.8 μM against *S. aureus*, 11.2 μM against *C. albicans*, and 22.4 μM against *E. coli* and *P. aeruginosa*. Cecropin A, an insect antimicrobial peptide, displays lethal concentrations of 0.32 μM against *E. coli* and 3.5 μM against *P. aeruginosa*[21]. PR-39, an AMP from pig intestine displays lethal concentrations of 0.3 μM against *E. coli* and 200 μM against *P. aeruginosa* and *S. aureus*[22]. The activity of the peptides was antagonised by the salt and divalent cations in a species-dependent manner. The outer membrane of Gram-negative bacteria contain lipopolysaccharide (LPS) as one of the major molecular components of the outer leaflet. The structure of LPS is characterized by a variable number of phosphate groups and anionic sugars in the oligosachharide core. The negatively charged groups are bridged by divalent cations[1,23]. The antimicrobial peptides bind the LPS by displacing the divalent cations. The structural diversity of the LPS contribute to the differential susceptibility of the Gram-negative bacteria to the antimicrobial peptides. There are many threatening Gram-positive bacteria, *S. aureus* being one of the most clinically-relevant ones. *S. aureus* is the most common bacterium present on human skin. It is considered an opportunistic pathogen but more aggressive strains have also evolved. *S. aureus* infections could be easily treated using Penicillin in 1940s[24]. Subsequently, *S. aureus* developed resistance against penicillin and could be

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treated with a Penicillin analog, Methicillin. A methicillin-resistant *S. aureus* strain was identified in 1960s and this was the birth of difficult to treat methicillin-resistant *S. aureus* (MRSA)[25]. It is arguably the most important hospital-acquired human pathogen. Although historically a hospital-acquired infection, community-acquired MRSA strains have emerged and are now epidemic in United States. It is very urgent to come up with new antimicrobials to treat MRSA infections. $MreB_{1-9}$ and its acetylated analog displayed lower activity against *S. aureus* compared to that against the Gram-negative bacteria. However, MRSA could be efficiently killed at concentrations comparable to those required for killing Gram-negative bacteria. N-terminal extension with a tryptophan residue drastically improves the activity wherein both methicillin susceptible and resistant bacteria could be killed at $\leq 10\,\mu M$ peptide concentrations. The most notable feature is that all the peptides retained >70% activity against *S. aureus* in the presence of salt and divalent cations. The peptides displayed excellent antifungal activity as well, killing *C. albicans* at concentrations as low as $5\,\mu M$. Candida is a clinically-important fungus and *C. albicans* happens to be the most common species causing invasive candidiasis[26]. All the four peptides could efficiently kill *C. albicans* and retained substantial activity in the presence of salt and divalent cations.

Conclusion

Interaction of proteins with membranes is an indispensable process for any life form and microbes are no exception. This study reports that the lipid-binding stretch of *E. coli* MreB could be developed into a potent, wide-spectrum antibiotic, killing both Gram-negative and Gram-positive bacteria as well as yeast. Development of the membrane-perturbing antimicrobial peptides from the membrane-binding stretches of microbial proteins, therefore, could be an excellent strategy to combat microbes especially the antibiotic-resistant ones. A self-like sequence would be less prone to enzymatic degradation by the bacteria. Furthermore, as these peptides kill the microbes by permeating/perturbing the cell membranes rather than targeting an intracellular target, they are less likely to induce resistance in the pathogens.

Materials and Methods

Materials. NovaPEG Rink amide resin, Fmoc-protected amino acids, and *N*,*N*,*N*',*N*'-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) were purchased from Novabiochem (Darmstadt, Germany). *N*,*N*-diisopropylethylamine, trifluoroacetic acid, ethanedithiol, thioanisole, piperidine, and acetic anhydride were from Sigma-Aldrich Chemicals Pvt. Ltd. 1-hydroxybenzotriazole hydrate, diethyl ether, and ethylenediaminetetraacetic acid disodium salt were from Sisco Research Laboratory, India. *N*,*N*-dimethylformamide and *m*-cresol were purchased from Merck India. Lipids were obtained from Avanti Polar Lipids. Media and supplements for growing bacteria and fungi were obtained from HiMedia. The bacterial strains used were *Escherichia coli* (MG 1655), *Pseudomonas aeruginosa* (NCTC 6750), *Salmonella enterica* (SL 4213), *Staphylococcus aureus* (NCTC 8530), and Gentamicin and Methicillin resistant *Staphylococcus aureus* (ATCC 33592) while *Candida albicans* (ATCC 18804) was used for the antifungal assay.

Peptides synthesis. Peptides were synthesized manually by solid-phase peptide synthesis using Fmoc chemistry. N-terminal acetylation was carried out on-resin using 5 equivalents of acetic anhydride and 10 equivalents of *N*,*N*-diisopropylethylamine. The peptides were cleaved from the resin using a cleavage cocktail comprising trifluoroacetic acid, ethanedithiol, thioanisole, and *m*-cresol (20:1:2:2). The peptides were precipitated in ice-cold diethyl ether and purified on Shimadzu Prominence Modular HPLC instrument on a reversed-phase C18 column using a linear gradient of water and acetonitrile (10–100%) containing 0.1% TFA. The purity of all the peptides was >95% as ascertained by analytical reversed-phase HPLC (see Supplementary Fig. S1). The peptides were characterized using matrix-assisted laser desorption/ionization mass spectrometry on a Bruker, Autoflex Speed MALDI TOF/TOF (see Supplementary Fig. S2). The stock solutions of 0.5–1 mM were prepared in deionized water and concentrations estimated using a molar absorption coefficient of $5690\,M^{-1}\,cm^{-1}$ at 280 nm for tryptophan-containing peptides and a molar absorption coefficient of $286\,M^{-1}\,cm^{-1}$ at 254 nm for the tryptophan-lacking peptides.

Surface activity and membrane binding. Surface activity and membrane binding studies were carried out on a KSV Nima Langmuir instrument (Biolin Scientific) using a custom made polytetrafluoroethylene trough of $13.2\,cm^2$ area. Surface pressure was measured by Wilhelmy method using a platinum plate (Biolin Scientific). Phosphate buffer (10 mM, pH 7.4) was used as the aqueous subphase. Peptides were injected into the subphase and increase in surface pressure was monitored over time. The maximal surface pressure change was plotted against the peptide concentration[27].

Lipids dissolved in chloroform were spread on the subphase to achieve initial surface pressure of $30–32\,mN\,m^{-1}$. The peptides were injected into the subphase to obtain a concentration of $10\,\mu M$ and mixing was achieved by magnetic stirring. The increase in surface pressure caused by the peptides was recorded as a function of time. The maximal changes in the surface pressure are reported.

Antimicrobial assay. Mid-log phase cells were harvested and washed twice with 10 mM phosphate buffer of pH 7.4. The cells were then diluted in the same buffer so as to have approximately 10^6 colony forming units (CFU)/mL. One hundred microliter of the cell suspensions were treated with different concentrations of the peptides (final volume adjusted to $120\,\mu L$) and incubated at 37 °C (bacteria) and 28 °C (fungus) for 2 hours. Following incubation, the cell suspensions were 10-fold diluted in 10 mM phosphate buffer, pH 7.4 and $20\,\mu L$ volumes were spread on nutrient agar plates (bacteria) and yeast extract-peptone-dextrose (YPD) agar plates (fungus). Bacterial plates were incubated at 37 °C for 12–18 hours while fungal plates were incubated at 28 °C for 24–30 hours. The colonies were counted to determine the antimicrobial activity. The peptide concentrations that resulted in complete killing of the microbes were considered the minimum lethal concentrations.

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Killing kinetics. The microbes were treated with the minimum lethal concentration of the peptides. The killing kinetics assay was performed by taking out 20 μL aliquots of the peptide-treated microbes after 1, 5, 15, 30, 60, and 120 minutes. The activity was determined as mentioned in the "Antimicrobial assay" section.

Salt sensitivity assay. Mid-log phase microbial cells were diluted in 10 mM phosphate buffer, pH 7.4 having 100 mM NaCl or 2 mM $CaCl_2$ or 1 mM $MgCl_2$. Peptides at their lethal concentrations were added to 100 μL microbial suspensions (final volume adjusted to 120 μL) and incubated at 37 °C (bacteria) and 28 °C (fungus) for 2 hours. Aliquots of 20 μL were 10-fold diluted and spread-plated. Colonies were counted to determine the activity.

Hemolytic assay. Human blood (~2 mL) was collected from a healthy individual in a tube containing ethylenediaminetetraacetic acid disodium salt and centrifuged at 800 × g for 5 minutes. The pellet was resuspended in 5 mM HEPES buffer, pH 7.4 containing 150 mM NaCl. Erythrocytes were washed several times and a 5% hematocrit was prepared in the same buffered saline. Peptides at 50 μM and 100 μM concentrations were incubated with 100 μL of 5% hematocrit and incubated for 1 hour at 37 °C. Following incubation, the hematocrit was centrifuged at 800 × g for 5 minutes and the absorbance of the supernatant was recorded at 540 nm. Hematocrit incubated with deionized water was considered as the positive control (100% lysis).

FESEM analysis. Mid-log phase cells (10^7) were treated with the minimal lethal concentrations of the peptides and incubated at 37 °C (bacteria) and 28 °C (fungus) for 2 hours. Incubated cells were centrifuged at 5000 × g for 5 minutes. The pellets were treated with 2.5% glutaraldehyde and incubated at 4 °C for 3 hours. Subsequently, cells were centrifuged at 5000 × g for 5 minutes and the supernatant was discarded. Cells were washed twice with phosphate buffer, loaded on a glass coverslip and dried at room temperature. The loaded samples were washed with deionized water and ethanol gradient ranging from 30–100%. The samples were air-dried, sputter-coated with gold, and analysed using FESEM.

Declaration. The experiments and methods associated with human blood were carried out in "accordance" with the relevant guidelines. All the experimental protocols were approved by the ethics committee of Indian Institute of Technology Guwahati. An informed consent was obtained from all subjects.

References

1. Zasloff, M. Antimicrobial peptides of multicellular organisms. *Nature* **415,** 389–395 (2002).
2. Cederlund, A., Gudmundsson, G. H. & Agerberth, B. Antimicrobial peptides important in innate immunity. *FEBS J.* **278,** 3942–3951 (2011).
3. Mishra, B. & Wang, G. Ab initio design of potent anti-MRSA peptides based on database filtering technology. *J. Am. Chem. Soc.* **134,** 12426–12429 (2012).
4. Fan, L. *et al.* DRAMP: a comprehensive data repository of antimicrobial peptides. *Sci. Rep.* **6,** 24482 (2016).
5. Jenssen, H., Hamill, P. & Hancock, R. E. Peptide antimicrobial agents. *Clin. Microbiol. Rev.* **19,** 491–511 (2006).
6. Huang, Y., Huang, J. & Chen, Y. Alpha-helical cationic antimicrobial peptides: relationships of structure and function. *Protein Cell* **1,** 143–152 (2010).
7. Giangaspero, A., Sandri, L. & Tossi, A. Amphipathic alpha helical antimicrobial peptides. *Eur. J. Biochem.* **268,** 5589–5600 (2001).
8. Tavares, L. S. *et al.* Strategies and molecular tools to fight antimicrobial resistance: resistome, transcriptome, and antimicrobial peptides. *Front. Microbiol.* **4,** 412 (2013).
9. Marr, A. K., Gooderham, W. J. & Hancock, R. E. Antibacterial peptides for therapeutic use: obstacles and realistic outlook. *Curr. Opin. Pharmacol.* **6,** 468–472 (2006).
10. Seo, M. D., Won, H. S., Kim, J. H., Mishig-Ochir, T. & Lee, B. J. Antimicrobial peptides for therapeutic applications: a review. *Molecules* **17,** 12276–12286 (2012).
11. Salje, J., van den Ent, F., de Boer, P. & Lowe, J. Direct membrane binding by bacterial actin MreB. *Mol. Cell* **43,** 478–487 (2011).
12. Gautier, R., Douguet, D., Antonny, B. & Drin, G. HELIQUEST: a web server to screen sequences with specific alpha-helical properties. *Bioinformatics* **24,** 2101–2102 (2008).
13. Keller, R. C. The prediction of novel multiple lipid-binding regions in protein translocation motor proteins: a possible general feature. *Cell Mol. Biol. Lett.* **16,** 40–54 (2011).
14. Ge, Y. *et al. In vitro* antibacterial properties of pexiganan, an analog of magainin. *Antimicrob. Agents Chemother.* **43,** 782–788 (1999).
15. Varkey, J. & Nagaraj, R. Antibacterial activity of human neutrophil defensin HNP-1 analogs without cysteines. *Antimicrob. Agents Chemother.* **49,** 4561–4566 (2005).
16. Craik, D. J., Fairlie, D. P., Liras, S. & Price, D. The future of peptide-based drugs. *Chem. Biol. Drug Des.* **81,** 136–147 (2013).
17. Waterer, G. W. & Wunderink, R. G. Increasing threat of Gram-negative bacteria. *Crit. Care Med.* **29,** N75–81 (2001).
18. Daxboeck, F., Budic, T., Assadian, O., Reich, M. & Koller, W. Economic burden associated with multi-resistant Gram-negative organisms compared with that for methicillin-resistant Staphylococcus aureus in a university teaching hospital. *J. Hosp. Infect.* **62,** 214–218 (2006).
19. Lister, P. D., Wolter, D. J. & Hanson, N. D. Antibacterial-resistant Pseudomonas aeruginosa: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. *Clin. Microbiol. Rev.* **22,** 582–610 (2009).
20. Zhou, C. *et al.* High potency and broad-spectrum antimicrobial peptides synthesized via ring-opening polymerization of alpha-aminoacid-N-carboxyanhydrides. *Biomacromolecules* **11,** 60–67 (2010).
21. Andreu, D., Merrifield, R. B., Steiner, H. & Boman, H. G. Solid-phase synthesis of cecropin A and related peptides. *Proc. Natl. Acad. Sci. USA* **80,** 6475–6479 (1983).
22. Agerberth, B. *et al.* Amino acid sequence of PR-39. *Eur. J. Biochem.* **202,** 849–854 (1991).
23. Torcato, I. M. *et al.* Design and characterization of novel antimicrobial peptides, R-BP100 and RW-BP100, with activity against Gram-negative and Gram-positive bacteria. *Biochim. Biophys. Acta* **1828,** 944–955 (2013).
24. Chambers, H. F. & Deleo, F. R. Waves of resistance: Staphylococcus aureus in the antibiotic era. *Nat. Rev. Microbiol.* **7,** 629–641 (2009).
25. Enright, M. C. *et al.* The evolutionary history of methicillin-resistant Staphylococcus aureus (MRSA). *Proc. Natl. Acad. Sci. USA* **99,** 7687–7692 (2002).
26. Lum, K. Y. *et al.* Activity of Novel Synthetic Peptides against Candida albicans. *Sci. Rep.* **5,** 9657 (2015).
27. Dennison, S. R., Baker, R. D., Nicholl, I. D. & Phoenix, D. A. Interactions of cell penetrating peptide Tat with model membranes: a biophysical study. *Biochem. Biophys. Res. Commun.* **363,** 178–182 (2007).

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Author Contributions

N.C. conceived the idea and the experiments. K.S. and Y.D.S. conducted the experiments. K.S. and N.C. analysed the results and wrote manuscript. N.C. and V.R. performed the Heliquest analysis of the peptides. All authors reviewed the manuscript.

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Interaction of MreB-derived antimicrobial peptides with membranes

Karabi Saikia, Nitin Chaudhary*

Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, 781 039, India

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ABSTRACT

Antimicrobial peptides are critical components of defense systems in living forms. The activity is conferred largely by the selective membrane-permeabilizing ability. In our earlier work, we derived potent antimicrobial peptides from the 9-residue long, N-terminal amphipathic helix of *E. coli* MreB protein. The peptides display broad-spectrum activity, killing not only Gram-positive and Gram-negative bacteria but opportunistic fungus, *Candida albicans* as well. These results proved that membrane-binding stretches of bacterial proteins could turn out to be self-harming when applied from outside. Here, we studied the membrane-binding and membrane-perturbing potential of these peptides. Steady-state tryptophan fluorescence studies with tryptophan extended peptides, $WMreB_{1\text{-}9}$ and its N-terminal acetylated analog, $Ac\text{-}WMreB_{1\text{-}9}$ show preferential binding to negatively-charged liposomes. Both the peptides cause permeabilization of *E. coli* inner and outer-membranes. Tryptophan-lacking peptides, though permeabilize the outer-membrane efficiently, little permeabilization of the inner-membrane is observed. These data attest membrane-destabilization as the mechanism of rapid bacterial killing. This study is expected to motivate the research in identifying microbes' self-sequences to combat them.

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1. Introduction

Indiscriminate use of antibiotics has led to the emergence of antibiotic-resistant microbes; multidrug-resistant and extensively-drug-resistant clinical strains pose a serious health threat. The gravity of the drug-resistance could be realized from the fact that many of the clinical strains are resistant to all antibiotics but one and many others are resistant to all [1]. Discovery of new class of antibiotics, therefore, is the pressing need in healthcare. Resistance to conventional antibiotics and slow discovery of new ones has revived the antimicrobial peptide research [2]. Antimicrobial peptides (AMPs) happen to be an important component of innate immune system in almost all life forms [3,4]. A large number of naturally-occurring AMPs have been identified from organisms ranging from bacteria to human. Peptides therefore constitute a natural and preferred class of antimicrobial agents. AMPs are very diverse in their length, sequence, and structure. Such diversity imparts them selectivity but makes it difficult to classify them based on their sequences or structures. Most AMPs, however, are cationic i.e. possess a net positive charge at neutral pH, a property that imparts preferential binding towards negatively-charged bacterial membranes [5]. Furthermore, most AMPs fold into amphipathic structures in membrane-like environment. Consequently, cationic AMPs exhibit their activity largely by selectively binding and permeabilizing the negatively-charged bacterial membranes. Peptides are interesting antimicrobial agents as they have survived the evolutionary pressure and continue to be used by all known organisms. Their activity could be modulated by careful editing of their sequences that includes incorporation of D-amino acids and other amino acid and non-amino acid analogs or by modifying their termini that imparts protection from proteolytic degradation [6]. Chemical diversity of the amino acids could be efficiently utilized to develop peptides of desired physicochemical properties.

Interaction of proteins with membranes is central to all living forms. We reasoned that the membrane-binding regions present in bacterial proteins could be developed as membrane-permeabilizing antimicrobial agents against them. The proof of concept was demonstrated using peptides derived from the membrane-binding region of *E. coli* MreB protein. In *E. coli* MreB, the N-terminal 9-residue stretch folds into an amphipathic helix and is sufficient and necessary for its binding with membrane [7]. We have demonstrated that the 9-residue stretch, in isolation, is a potent antimicrobial peptide. The MreB-derived peptides efficiently killed not only *E. coli* but Gram-positive bacteria and *C. albicans* as well [8]. In this study, we investigated the interaction

* Corresponding author.
E-mail address: chaudhary@iitg.ernet.in (N. Chaudhary).

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of these peptides with model membranes. In addition to that, permeabilization of bacterial membranes was studied using *E. coli* as the model microorganism.

2. Materials and methods

2.1. Materials

Reagents for peptide synthesis were procured from Novabiochem, Sigma Aldrich, Merck, and Sisco Research Laboratory as mentioned earlier [8]. 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), and cholesterol (CHL) were obtained from Avanti Polar Lipids. Polymyxin B, 1-N-phenylnaphthylamine (NPN), 3,3′-dipropylthiadicarbocyanine iodide ($DiSC_3(5)$) and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Aldrich. Bacterial growth medium was obtained from HiMedia. *Escherichia coli* (MG 1655) was used for the membrane permeabilization assays.

2.2. Peptide synthesis and characterization

Peptides were synthesized on NovaPEG Rink amide resin using Fmoc chemistry, purified using reversed-phase high performance liquid chromatography, and characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry as described previously [8]. The peptides were lyophilized and stored at −20 °C until further use. Stock solutions were prepared in deionized water and concentrations estimated as described previously [8].

2.3. Preparation of small unilamellar vesicles

Small unilamellar vesicles (SUVs) were prepared with POPE:POPG (7:3) and POPC:CHL (10:1) lipid compositions. Lipids in desired molar ratio were mixed and dried from stock solutions prepared in chloroform. Chloroform was evaporated using nitrogen gas to obtain thin lipid films. The dried lipids were desiccated for six hours and then hydrated overnight using 10 mM HEPES buffer, pH 7.4 containing 150 mM NaCl and 0.1 mM EDTA (HEPES-buffered saline, HBS). Following hydration, lipid suspension was vigorously vortexed and sonicated in a water bath sonicator till clear lipid suspensions were obtained [9].

2.4. Steady state tryptophan fluorescence

Tryptophan fluorescence spectra were recorded for the peptides (1 μM) with and without SUVs in HBS. With SUVs, the spectra were recorded for 1:50 and 1:100 peptide:lipid molar ratios. The spectra were recorded on FP-8500 spectrofluorometer (Jasco) by exciting the samples at 280 nm and recording emission from 300 to 450 nm. Excitation and emission slit widths were 2.5 and 5 nm, respectively. The spectra were corrected by subtracting the corresponding blanks i.e. samples without peptides.

2.5. Tryptophan fluorescence quenching

Peptide solutions (1 μM) in HBS, without SUVs and with SUVs (peptide:lipid ratio of 1:100) were excited at 295 nm and fluorescence emission spectra recorded from 300 to 450 nm. The samples were subsequently titrated with increasing amounts of acrylamide in the range of 0.02 M–0.14 M and fluorescence spectra were recorded. Excitation and emission slit widths were 2.5 and 5 nm, respectively. The spectra were corrected by subtracting the spectra obtained from corresponding blanks *i.e.* the samples without peptides. Fluorescence intensity values at 350 nm were extracted and data were analyzed using Stern-Volmer equation, $F_0/F = 1 + K_{SV}[Q]$, where F_0 is the fluorescence in the absence of quencher, F is the fluorescence intensity in the presence of quencher, K_{SV} is the Stern-Volmer constant, and [Q] is the quencher concentration [9].

2.6. Outer-membrane permeabilization assay

The assay was carried out with *E. coli* cells using non-polar probe, NPN. Cells were grown to mid-log phase, harvested, washed with 5 mM HEPES buffer, pH 7.4 having 5 mM glucose, and diluted to obtain an optical density of 0.5 at 600 nm. Subsequently, NPN was added to obtain a final concentration of 10 μM and fluorescence emission monitored at 420 nm by exciting at 350 nm. The excitation and emission slit widths were 2.5 and 5 nm, respectively. Peptides at different concentrations were added and fluorescence recorded as a function of time until no further increase in fluorescence is observed. Polymyxin B (10 μg/ml) was used as the positive control. Percentage NPN uptake is calculated using equation: $\%\ NPN = [(F_{obs} - F_0)/(F_{100} - F_0)] \times 100$, where F_{obs} is the observed fluorescence at a given peptide concentration, F_0 is the initial fluorescence of NPN with *E. coli* cells in the absence of peptide and F_{100} is the fluorescence of NPN with *E. coli* cells in the presence of 10 μg/ml polymyxin B [9].

2.7. Inner-membrane permeabilization assay

Mid-log phase *E. coli* cells were washed with 5 mM HEPES buffer, pH 7.4 having 20 mM glucose. Bacterial optical density was adjusted to 0.05 at 600 nm. $DiSC_3(5)$ was added to the cell suspension to obtain a 0.4 μM $diSC_3(5)$ and incubated for 1 h so that the dye gets integrated into the inner-membrane. KCl (100 mM) was added to the culture to equilibrate potassium concentration inside and outside the membrane. Fluorescence emission was recorded at 670 nm by exciting the sample at 622 nm. The excitation and emission slit widths were 2.5 and 5 nm, respectively. Peptides were added at different concentrations and change in fluorescence intensity recorded as a function of time [9].

2.8. Circular dichroism spectroscopy

Circular dichroism (CD) spectra were recorded on Jasco J-1500 spectropolarimeter in a 1 mm path length cell with a slit width of 1 nm. Spectra were recorded for peptides (50 μM) in trifluoroethanol (TFE) and in 10 mM phosphate buffer, pH 7.4 having 100 mM NaCl and 0.1 mM EDTA without and with SUVs (500 μM lipid concentration). Each of the $MreB_{1\text{-}9}$ spectra is the average of 16 scans whereas all other spectra are average of 8 scans. The spectra are smoothed using Savitzky-Golay algorithm [10,11] and presented as mean residue ellipticity, $[\theta]_{MRE}$. Mean residue ellipticity was calculated using the formula: $[\theta]_{MRE} = (M_r \times \theta_{mdeg})/(10 \times l \times c)$, where M_r is the mean residue weight *i.e.* peptide molecular weight/number of residues, θ_{mdeg} is the ellipticity in millidegrees, l is the length in centimeters, and c is the peptide concentration in mg/ml [12].

3. Results

Antimicrobial properties of the four *E. coli* MreB protein-derived peptides have been reported earlier [8]. The sequences of the peptides are shown in Table 1.

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Table 1
Sequences and net charge on the peptides.

Peptide	Amino acid sequence	Net charge at pH 7.4
$MreB_{1-9}$	NH_2-MLKKFRGMF-am	+4
Ac-$MreB_{1-9}$	Ac-MLKKFRGMF-am	+3
$WMreB_{1-9}$	NH_2-WMLKKFRGMF-am	+4
Ac-$WMreB_{1-9}$	Ac-MLKKFRGMF-am	+3

'Ac-' represents amino-terminal acetylation, while '-am' represents C-terminal amide.

3.1. Steady state tryptophan fluorescence

Binding of $WMreB_{1-9}$ and Ac-$WMreB_{1-9}$ to liposomes was studied using tryptophan fluorescence. In HBS, the peptides display fluorescence emission band centered around 353 nm suggesting solvent exposed tryptophan side-chain (Fig. 1).

In the presence of POPC:CHL SUVs, both the peptides display emission maxima ~346–348 nm at 1:100 peptide/lipid ratio. The little blue shift in the emission spectra suggests binding to liposomes. In the presence of POPE:POPG SUVs, Ac-$WMreB_{1-9}$ displays emission maxima ~345 nm and ~343 nm for peptide/lipid ratios of 1:50 and 1:100, respectively. $WMreB_{1-9}$, on the other hand displays emission maxima ~340 nm at both the peptide/lipid ratios. Large blue shift observed for $WMreB_{1-9}$ with negatively-charged vesicles could be attributed to the charged amino-terminus. The data clearly suggest preferential binding towards negatively-charged membranes for both the peptides.

3.2. Tryptophan fluorescence quenching

Accessibility of tryptophan to dynamic quencher, acrylamide was studied. Fig. 2 shows the Stern-Volmer plots of $WMreB_{1-9}$ and Ac-$WMreB_{1-9}$ in the absence and presence of lipid vesicles.

The tryptophan side chains display high solvent accessibility for both the peptides in HBS which is in agreement with the emission maxima data in Fig. 1. For the peptides in HBS, the Stern-Volmer plots deviate from linearity at concentrations higher than 0.06 M acrylamide. The Stern-Volmer constants, K_{SV} for the peptides in HBS were, therefore calculated using the linear region *i.e.* from 0 to 0.06 M acrylamide concentration range (Table 2). Stern-Volmer plots for the peptides in the presence of SUVs fit linearly and the K_{SV} values determined are shown in Table 2. The extent of quenching is considerably less in the presence of SUVs. The net accessibility factors (NAF) were calculated using the formula, NAF = K_{SV} (with SUVs)/K_{SV} (without SUVs). The data show that the tryptophan side-chain is largely inaccessible to the quencher in the presence of negatively-charged lipid vesicles.

3.3. Outer-membrane permeabilization assay

Permeabilization of *E. coli* outer-membrane was studied using NPN fluorescence. NPN is a fluorophore that weakly fluoresces in aqueous solutions but exhibits enhanced fluorescence in hydrophobic environments. Perturbation of lipid bilayer allows NPN an access to the hydrophobic environment thereby causing an enhancement in its fluorescence. Polymyxin B is a known outer-membrane permeabilizing agent that is used as a positive control in the assay. Table 3 shows outer-membrane permeabilizing ability of the MreB-derived peptides relative to Polymyxin B.

All the four peptides cause permeabilization of *E. coli* outer-membrane. The maximal NPN uptake (~97%) for Ac-$MreB_{1-9}$ was obtained at 15 μM peptide concentration. For other three peptides, 100% uptake could be obtained at 10 μM concentration.

3.4. Inner-membrane permeabilization assay

$DiSC_3(5)$ is a membrane-potential sensitive fluorophore. The dye partitions to the surface of polarized cells, gets concentrated and consequently self-quenched [13]. Disruption of membrane potential releases the dye into the medium thereby causing an increase in its fluorescence. $MreB_{1-9}$ and Ac-$MreB_{1-9}$ did not cause enhancement in fluorescence (Fig. 3). Rather, the fluorescence intensity was dramatically reduced for $MreB_{1-9}$. This decrease could be attributed to outer-membrane permeabilization that allows $diSC_3(5)$ a ready access to inner-membrane. At 15 and 20 μM concentrations, however, the decrease in fluorescence is followed by a small increase, suggesting disruption of membrane potential. $WMreB_{1-9}$ and Ac-$WMreB_{1-9}$, on the other hand, rapidly permeablize the inner-membrane causing immediate enhancement in fluorescence intensity.

3.5. Circular dichroism spectroscopy

CD spectroscopic analysis shows that the peptides are unordered in 10 mM phosphate buffer, pH 7.4 having 100 mM NaCl and 0.1 mM EDTA (Fig. 4).

In the presence of POPC:CHL SUVs, $WMreB_{1-9}$ becomes

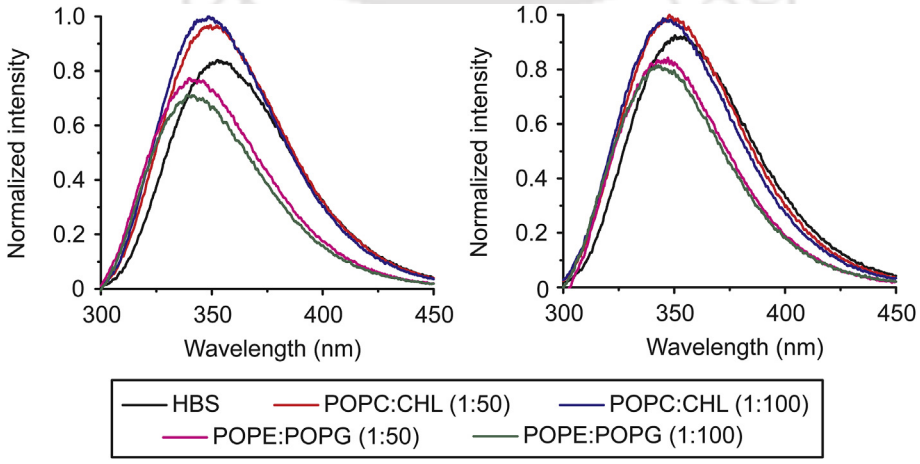

Fig. 1. Tryptophan fluorescence emission spectra of $WMreB_{1-9}$ (left panel) and Ac-$WMreB_{1-9}$ (right panel). The spectra were recorded in the absence of lipid (black trace) and in the presence of SUVs such that the peptide/lipid ratios are 1:50 and 1:100.

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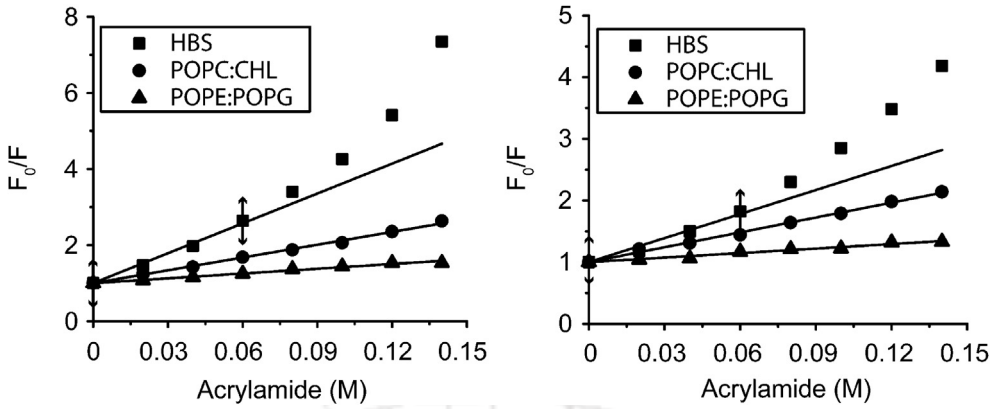

Fig. 2. Stern-Volmer plots showing the tryptophan fluorescence quenching by acrylamide in $WMreB_{1-9}$ (left panel) and Ac-$WMreB_{1-9}$ (right panel) peptides in HBS (■), POPC:CHL SUVs(●), and POPE:POPG SUVs (▲). The vertical arrows indicate the concentration range of acrylamide used for fitting the data for peptides in HBS.

Table 2
Ksv and NAF values of the MreB-derived peptides.

	HBS		POPC:CHL		POPE:POPG	
	K_{SV} (M^{-1})	NAF	K_{SV} (M^{-1})	NAF	K_{SV} (M^{-1})	NAF
$WMreB_{1-9}$	26.2	1	11.2	0.43	4.2	0.16
Ac-$WMreB_{1-9}$	13.0	1	8.0	0.61	2.4	0.18

structured while other three peptides are largely unordered (panel C). All the four peptides show positive ellipticity ~195–197 nm in the presence of POPE:POPG SUVs, suggesting folding of the peptides. The spectrum for $MreB_{1-9}$ in the presence of POPE:POPG vesicles show negative bands around 222 and 205 nm and a weak positive band ~197 nm. The spectrum is suggestive of a mixture of α-helical and random coil conformations (panel A). Ac-$MreB_{1-9}$ and W-$MreB_{1-9}$ fold into distinct α-helical conformation in the presence of negatively charged SUVs (panels B and C). Ac-$WMreB_{1-9}$ shows a negative band centered ~226 nm and a positive band centered ~195 nm suggesting a mixture of α-helical and β-strand conformations. The data suggests that the peptides are unstructured in water but fold upon binding to negatively-charged lipid vesicles. In TFE, $MreB_{1-9}$ spectrum suggests a mixture of α-helical and random coil conformations (panel A). Ac-$MreB_{1-9}$, $WMreB_{1-9}$, and Ac-$WMreB_{1-9}$, on the other hand, take up distinct α-helical conformation (panels B-D).

4. Discussion

It is interesting that almost all known organisms utilize peptides to combat pathogens. A large number of antimicrobial peptides have been isolated from various organisms. The naturally-occurring antimicrobial peptides are highly diverse in their sequences and structures. In view of the menace of multidrug-resistant microbes and slow discovery of new antibiotics, the peptides and peptidomimetics are being looked at with great hope. We hypothesized that bacterial proteins that interact with their membranes could be developed into membrane-permeabilizing antimicrobials and showed that the peptides derived from N-terminal membrane-binding region of *E. coli* MreB protein, efficiently kill Gram-negative bacteria, Gram-positive bacteria, and *C. albicans* [8].

Antibiotics discovered in last five decades are all active against Gram-positive bacteria. Gram-negative bacteremia, therefore, is a serious clinical issue. *Enterobacteriaceae* and *P. aeruginosa* are among the primary causative agents of nosocomial bloodstream infections. Emergence of multidrug-resistant Gram-negative superbugs further adds to the woe. Extensively-drug-resistant and pandrug-resistant clinical strains of *Klebsiella pneumoniae* and *P. aeruginosa* have been isolated from patients [1]. MreB-derived peptides, $WMreB_{1-9}$ and Ac-$WMreB_{1-9}$ have been shown to kill *E. coli*, *P. aerugionosa,* and *S. enterica* at very promising concentrations (≤10 μM) [8]. Both the peptides cause complete killing of these organisms within 5 min of peptide treatment. Ac-$WMreB_{1-9}$ is very promising as it kills these bacteria within 5 min of treatment. Furthermore, its activity is not compromised in the presence of salt and divalent cations. Such rapid killing is little likely to be mediated through inhibition of a protein receptor. Our results establish membrane-binding and membrane-permeabilizing potential of these peptides. The peptides preferentially bind to the negatively-charged vesicles as suggested by the tryptophan fluorescence studies of $WMreB_{1-9}$ and Ac-$WMreB_{1-9}$. NPN uptake by the peptide-treated *E. coli* cells suggests permeabilization of outer-membrane. Even though $MreB_{1-9}$ and Ac-$MreB_{1-9}$ caused little dissipation of inner-membrane potential, $WMreB_{1-9}$ and Ac-$WMreB_{1-9}$ efficiently dissipated the potential suggesting inner-membrane perturbation by these peptides. Nosocomial infections caused by multidrug-resistant Gram-negative bacteria usually need to be treated with polymyxins [14]. Polymyxins exhibit their activity by permabilizing the outer and inner-membranes of bacteria.

Table 3
Outer-membrane permeabilization of *E. coli* cells by MreB-derived peptides. The percentage NPN uptake was determined relative to that caused by 10 μg/ml polymyxin B.

Peptide concentration (μM)	$MreB_{1-9}$	Ac-$MreB_{1-9}$	$WMreB_{1-9}$	Ac- $WMreB_{1-9}$
	Percentage NPN uptake			
5	93.06 ± 9.41	52.08 ± 0.93	82.83 ± 0.7	83.16 ± 17.7
10	100	79.14 ± 18.05	100	100
15	100	97.31 ± 3.8	100	100

All the four peptides cause permeabilization of *E. coli* outer-membrane. The maximal NPN uptake (~97%) for Ac-MreB1-9 was obtained at 15 μM peptide concentration. For other three peptides, 100% uptake could be obtained at 10 μM concentration.

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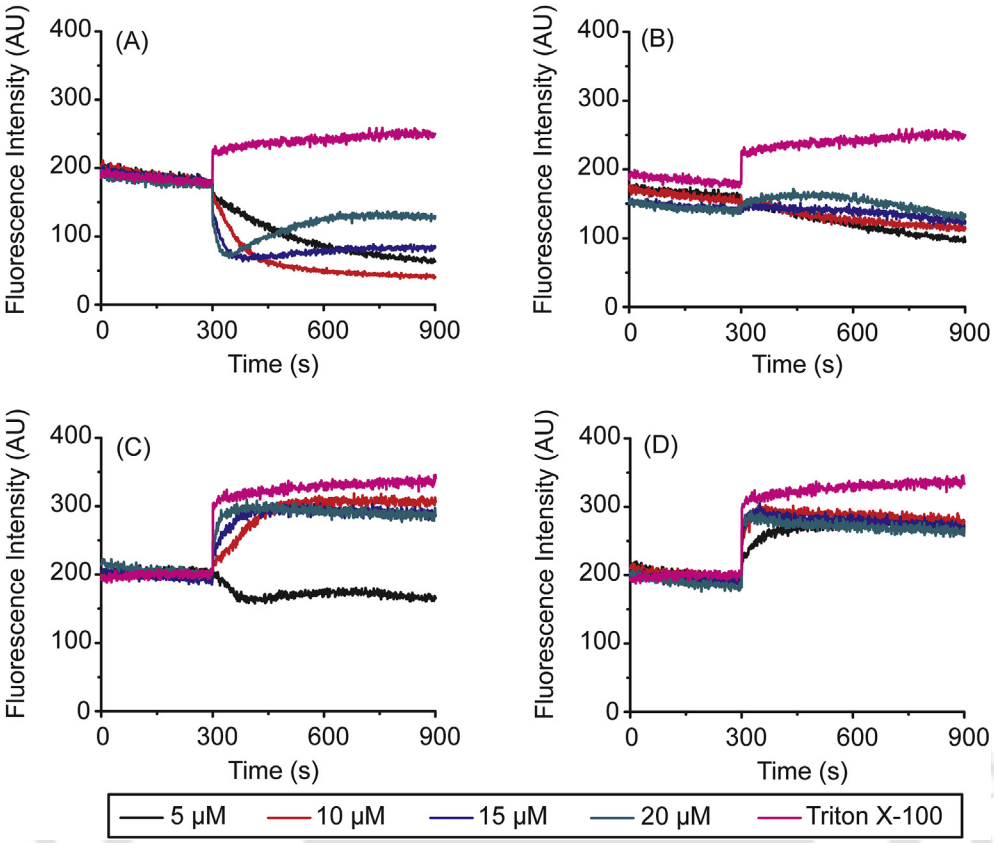

Fig. 3. Inner-membrane depolarization of *E. coli* cells by the MreB-derived peptides. An increase in fluorescence intensity of voltage-sensitive dye $diSC_3(5)$ following peptide treatment suggests depolarization of the inner-membrane. Panels A, B, C, and D correspond to $MreB_{1-9}$, Ac-$MreB_{1-9}$, $WMreB_{1-9}$, and Ac-$WMreB_{1-9}$, respectively.

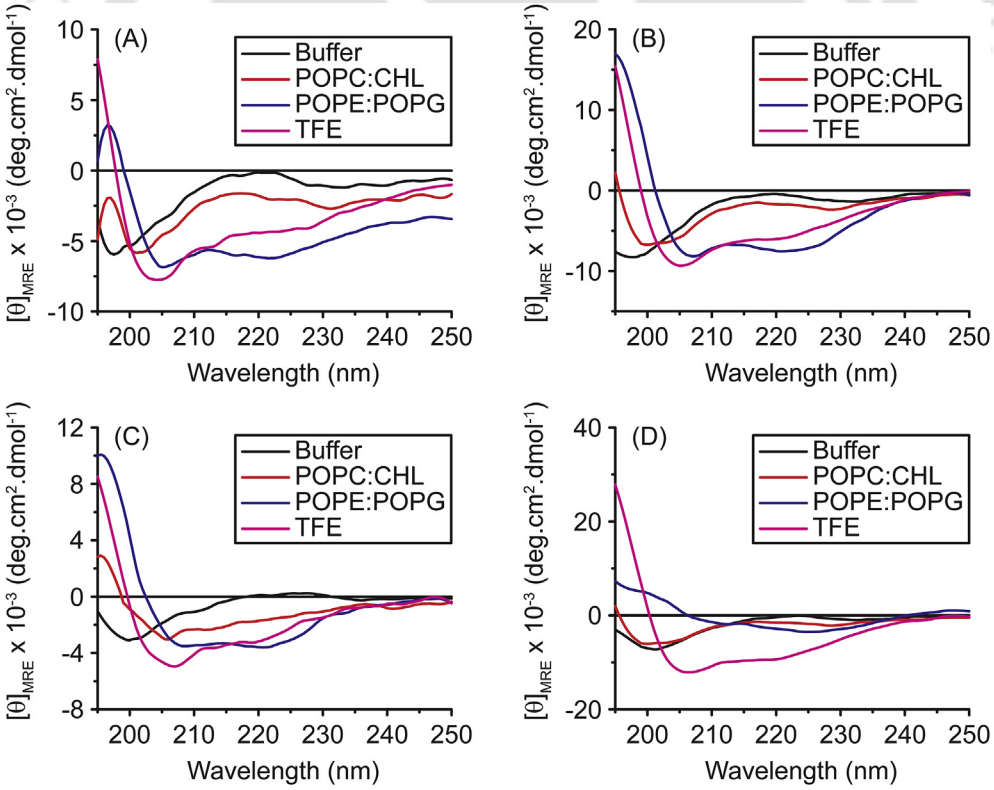

Fig. 4. Circular dichroism spectra of the peptides in 10 mM phosphate buffer, pH 7.4 having 100 mM NaCl and 0.1 mM EDTA without and with lipid SUVs. Panels A, B, C, and D correspond to $MreB_{1-9}$, Ac-$MreB_{1-9}$, $WMreB_{1-9}$, and Ac-$WMreB_{1-9}$, respectively.

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In this view, MreB-derived peptides hold the promise to treat Gram-negative bacterial infections. Potential to kill Gram-positive bacteria and *C. albicans* is an added advantage [8].

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References

[1] M.E. Falagas, I.A. Bliziotis, S.K. Kasiakou, G. Samonis, P. Athanassopoulou, A. Michalopoulos, Outcome of infections due to pandrug-resistant (PDR) Gram-negative bacteria, BMC Infect. Dis. 5 (2005) 24.
[2] J.L. Fox, Antimicrobial peptides stage a comeback, Nat. Biotechnol. 31 (2013) 379–382.
[3] H.M. Sergio, A. Gloria, Antimicrobial peptides: a natural alternative to chemical antibiotics and a potential for applied biotechnology, Electron. J. Biotechnol. 6 (3) (2003).
[4] A. Tossi, L. Sandri, A. Giangaspero, Amphipathic, alpha-helical antimicrobial peptides, Biopolymers 55 (2000) 4–30.
[5] M. Zasloff, Antimicrobial peptides of multicellular organisms, Nature 415 (2002) 389–395.
[6] M.D. Seo, H.S. Won, J.H. Kim, T. Mishig-Ochir, B.J. Lee, Antimicrobial peptides for therapeutic applications: a review, Molecules 17 (2012) 12276–12286.
[7] J. Salje, F. van den Ent, P. de Boer, J. Lowe, Direct membrane binding by bacterial actin MreB, Mol. Cell. 43 (2011) 478–487.
[8] K. Saikia, Y.D. Sravani, V. Ramakrishnan, N. Chaudhary, Highly potent antimicrobial peptides from N-terminal membrane-binding region of E. coli MreB, Sci. Rep. 7 (2017) 42994.
[9] Y. Lv, J. Wang, H. Gao, Z. Wang, N. Dong, Q. Ma, A. Shan, Antimicrobial properties and membrane-active mechanism of a potential alpha-helical antimicrobial derived from cathelicidin PMAP-36, PLoS One 9 (2014), e86364.
[10] A. Savitzky, M.J.E. Golay, Smoothing and differentiation of data by simplified least squares procedures, Anal. Chem. 36 (1964) 1627–1639.
[11] N.J. Greenfield, Using circular dichroism spectra to estimate protein secondary structure, Nat. Protoc. 1 (2006) 2876–2890.
[12] R. Pain, Determining the CD spectrum of a protein, Curr Protoc Protein Sci Chapter 7 (2005). Unit 7 6.
[13] M. Wu, R.E. Hancock, Interaction of the cyclic antimicrobial cationic peptide bactenecin with the outer and cytoplasmic membrane, J. Biol. Chem. 274 (1999) 29–35.
[14] A.S. Michalopoulos, D.C. Karatza, Multidrug-resistant Gram-negative infections: the use of colistin, Expert Rev. Anti Infect. Ther. 8 (2010) 1009–1017.

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Antimicrobial peptides from C-terminal amphipathic region of *E. coli* FtsA

Karabi Saikia, Nitin Chaudhary*

Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati 781 039, India

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ABSTRACT

Antimicrobial peptides constitute an indispensable component of innate immune system in organisms ranging from bacteria to man. Despite this, peptides lag far behind the conventional antibiotics in treating infections. The menace of multidrug-resistant bacteria, however, has revived the antimicrobial peptide research. We reasoned that the membrane-binding regions of bacterial proteins could be purposed to combat them. Here, we identify potent antimicrobial peptides from the C-terminal amphipathic helix of *E. coli* FtsA protein. The 11 and 13-residue peptides exhibited activity against *E. coli*, gentamicin-resistant MRSA, and *C. albicans*. The activity is little affected by the presence of salt and divalent cations. The peptides preferentially bind to the negatively-charged membranes as indicated by tryptophan fluorescence studies. The peptides permeabilize the *E. coli* outer and inner membranes at very promising concentrations suggesting membrane-disruption as one of the mechanisms of killing.

1. Introduction

Antimicrobial peptides (AMPs) are endogenous peptides produced by almost all life forms as an essential component of their innate immune system to fend off pathogens [1–4]. More than 2000 antimicrobial peptides have been isolated from very diverse range of organisms. AMPs typically comprise 10–50 residues, possess a net positive charge at neutral pH, and can fold into a variety of structures. Amphipathic α-helical peptides constitute the most abundant and widespread structural class of AMPs [5,6]. The sequences of AMPs are so diverse that it is difficult to classify them based on their sequences. The positive charge imparts them selectivity towards negatively-charged bacterial membranes. Folding in the membrane-like environment results in an amphipathic structure that disrupts bacterial membrane [7,8]. Membrane-disruption underlies the rapid killing of the microbe. In addition to that, disruption of membrane allows AMPs ready access to the intracellular targets as well. Binding of peptides specifically/non-specifically to intracellular targets would disturb the microbial homeostasis resulting in their death. Their ubiquitous nature, direct and rapid killing of the microbes, and slower discovery of novel antibiotics to combat drug-resistant bacteria has revived interest in the antimicrobial peptide research. The lessons learnt from the working principles of natural AMPs have been utilized to design novel AMPs with similar or better antimicrobial and pharmaceutical properties [6,9–13].

Protein-membrane interaction is central to life. We reasoned that membrane-binding regions of microbial proteins could turn out to be potent antimicrobials. Deriving antimicrobial peptides from membrane-binding regions of the infectious microbes could be an interesting strategy; such peptides would inhibit the very same microbe through membrane permeabilization and possibly by interfering with the normal function of the parent protein. The proof of concept was demonstrated with the AMPs derived from N-terminal membrane-binding region of *E. coli* MreB [14,15]. To substantiate the antimicrobial potential of the bacterial membrane-interacting stretches, we extended the study to another bacterial protein, FtsA. As most cationic AMPs display selectivity towards Gram-positive bacteria, it would be interesting to identify AMPs against Gram-negative bacteria. Malanovic and Lohner have carried out statistical analysis of the known AMPs to understand the AMP selectivity towards bacteria [16]. The analysis indicates a correlation between the peptide hydrophobicity and bacterial selectivity. Peptides that act against Gram-negative bacteria are less hydrophobic (≤50% hydrophobic residues) [16]. The conserved C-terminal amphipathic helix of *E. coli* FtsA protein was selected for its low hydrophobicity and high amphipathicity. FtsA, an abbreviation for filamentous temperature sensitive A, is a bacterial protein present in a majority of Gram-positive and Gram-negative bacteria. Structurally, FtsA resembles actin-like filaments and assist in cell division [17]. During cell division FtsA protein helps FtsZ polymers to tether to the cytoplasmic membrane with the help of a conserved C-terminal amphipathic helix [18]. The absence of helix in bacteria leads to the formation of long and stable polymer bundles of FtsA in the cells that cannot take part in cell division [19]. We investigated the antimicrobial

* Corresponding author.
E-mail address: chaudhary@iitg.ac.in (N. Chaudhary).

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properties of the 10, 11, and 13-residue peptides derived from FtsA protein against *E. coli*, *S. enterica*, *P. aeruginosa*, gentamicin-resistant *S. aureus* (gentamicin-resistant MRSA), and *C. albicans*. The peptides display preferential binding to the negatively-charged lipid vesicles and kill Gram-negative and Gram-positive bacteria as well as *C. albicans*. Permeabilization of the membrane(s) is suggested as one of the mechanisms of action for the peptides. Interference with intracellular molecules, even though not explored in this study, is very much possible.

2. Material and methods

2.1. Material

NovaPEG Rink amide resin, *N,N,N′,N′*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), and Fmoc-protected amino acids were procured from Novabiochem (Darmstadt, Germany). 1-hydroxybenzotriazole (HOBt) and diethyl ether were from SRL, India. *N,N*-diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA), *m*-cresol, thioanisole, ethanedithiol, acetic anhydride, chloroform, 1-phenyl naphthylamine (NPN), polymyxin B, ethylenediaminetetraacetic acid potassium salt (EDTA) were procured from Sigma-Aldrich Chemicals Pvt. Ltd. *N,N*-dimethylformamide, piperidine, and 3,3′-dipropylthiadicarbocyanine iodide ($DiSC_3(5)$) were obtained from Merck. Lipids were purchased from Avanti Polar Lipids. Media for growing bacteria and yeast, agar, and acrylamide were from Himedia. *Escherichia coli* (MG 1655), *Salmonella enterica* (SL 4123), *Pseudomonas aeruginosa* (NCTC 6750), gentamicin-methicillin-resistant *Staphylococcus aureus* (ATCC 33592), and *Candida albicans* (ATCC 18804) were used for antimicrobial assays.

2.2. Peptide synthesis

Peptides were synthesized using solid phase peptide synthesis by employing Fmoc chemistry with HBTU/HOBt activation as previously described [14]. The peptides were purified on a reversed-phase C18 column. The masses of the peptides were ascertained using MALDI-TOF mass spectrometry on Bruker Autoflex speed MALDI TOF/TOF.

2.3. Preparation of small unilamellar vesicles (SUVs)

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (sodium salt) (POPG), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), and cholesterol (CHL) were used in the preparation of SUVs. POPC:CHL (10:1) and POPE:POPG (7:3) SUVs were prepared in 10 mM HEPES buffer, pH 7.4 containing 150 mM NaCl and 0.1 mM EDTA (HEPES-buffered saline or HBS) as described previously [15].

2.4. Steady-state tryptophan fluorescence

Steady-state tryptophan fluorescence spectra for the peptides (1 μM) were recorded in HEPES-buffered saline without and with SUVs at the peptide to lipid molar ratios of 1:50 and 1:100 as previously described [15].

2.5. Tryptophan fluorescence quenching

Steady-state tryptophan fluorescence spectra for the peptides (1 μM) were recorded in HEPES-buffered saline in the presence of SUVs (peptide:lipid ratio of 1:100). Fluorescence quenching by acrylamide was studied as previously described [15]. The experiment was carried out in three replicates, and the representative data are shown.

2.6. Minimum inhibitory concentration of the peptides

Minimum inhibitory concentration (MIC) of the peptides was determined using broth microdilution method [20]. Fresh bacterial colonies were inoculated in Müller Hinton broth. Cultures were allowed to grow at 37 °C in a shaker incubator at 200 rpm to attain turbidity that is equal to McFarland standard 0.5 (0.08–0.1 OD at 625 nm). Cultures having higher turbidity were diluted with the media to obtain desired turbidity. MIC was determined using broth microdilution method in polypropylene microtiter plates. Two-fold concentrated peptides were serially diluted from a range of 128 μM to 1 μM in microtiter wells; the final concentration of cultures in the wells was 5×10^5 CFU/ml. Growth control and sterility control were included for the reliability of the experiment. Microtiter plates were incubated at 37 °C for 16–20 h and turbidity was examined with unaided eyes. MIC is the lowest concentration of peptide that inhibits the growth of microbes resulting in clear wells in the microtiter plates. For yeast, MIC is determined by broth microdilution method as per Clinical and Laboratory Standards Institute (CLSI) M27-A3 manual. Briefly, 24 h-old colonies were inoculated in 10 mM phosphate buffer pH 7.4 and the cell density was adjusted to that of 0.5 McFarland standard at 530 nm. The final dilution of cells was carried out in RPMI medium, buffered with 10 mM phosphate, pH 7.4 and supplemented with glutamine. Two-fold concentrated peptides were serially diluted in RPMI medium from a range of 128 μM to 2 μM in microtiter wells; the final concentration of cultures in the wells was 0.5×10^3–2.5×10^5 CFU/ml. Microtiter plates were incubated at 30 °C for 24 h and observed for the presence or absence of visible growth. The experiment was carried out in three replicates, and same MIC values were observed in each replicate.

2.7. Salt sensitivity assay

Sensitivity of the peptides to 100 mM NaCl, 1 mM $MgCl_2$ and 2 mM $CaCl_2$ was ascertained by determining the MIC values in the presence of these salts. MICs were determined as described above; a change in MIC indicates sensitivity to the salt. The experiment was carried out in three replicates, and same MIC values were observed in each replicate.

2.8. Outer membrane permeabilization assay

Outer-membrane permeabilization was assayed using NPN, a non-polar probe that exhibits enhanced fluorescence in hydrophobic environment. An increase in fluorescence intensity indicates outer-membrane permeabilization. Mid-log phase *E. coli* cells were harvested and washed twice with HEPES buffer, pH 7.4 containing 5 mM glucose. The cells were diluted in the same buffer to achieve an optical density of 0.5 at 600 nm. Subsequently, NPN (10 μM assay concentration) was added and fluorescence emission recorded at 420 nm (slit width = 5 nm) by exciting the samples at 350 nm (slit width = 2.5 nm). Peptides were added and fluorescence emission intensity recorded until no further increase in fluorescence intensity is obtained. The fluorescence intensity saturation reached within 10 min for all the peptides. Polymyxin B (10 μg/ml) served as positive control in the assay. Percentage NPN uptake with respect to that caused by polymyxin B was calculated using the relation: $\%NPN\ uptake = [(F_{obs} - F_0)/(F_{100} - F_0)] \times 100$, where F_{obs} is the final fluorescence intensity, F_0 is fluorescence intensity in the absence of peptide, and F_{100} is the final NPN fluorescence intensity in the presence of polymyxin B [15]. The experiment was carried out in three replicates and mean NPN uptake with standard deviation is reported. Interaction of peptides with NPN was examined and none of the peptides was found to interfere with its fluorescence.

2.9. Inner membrane depolarization assay

Cytoplasmic membrane depolarization of *E. coli* cells was studied using voltage-sensitive dye $diSC_3(5)$. Mid-log phase bacterial cells in

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5 mM HEPES buffer, pH 7.4 with 20 mM glucose were adjusted to an optical density of 0.05 at 600 nm. Subsequently, the cells were treated with EDTA (0.2 mM) for causing permeabilization of the outer membrane to facilitate the dye uptake. The cells were then treated with $diSC_3(5)$ such that the final dye concentration is 0.4 μM and incubated for 1 h. Subsequently, KCl (100 mM) was added to equilibrate potassium ion concentration inside and outside the membrane [21]. The samples were excited at 622 nm (slit width = 1 nm) and fluorescence emission recorded at 670 nm (slit width = 5 nm). Peptides (8 μM assay concentration) were added and changes in the fluorescence intensity measured as a function of time. Polymyxin B (10 μg/ml) served as the positive control in the assay. The experiment was carried out in three replicates and representative data are shown. None of the peptides was found to interfere with $diSC_3(5)$ fluorescence as examined by recording $diSC_3(5)$ fluorescence with peptides.

2.10. Field-emission scanning electron microscopy (FESEM) analysis

Mid-log phase microbial cells were used for the FESEM imaging. Cells were washed with 10 mM phosphate buffer, pH 7.4 and the cell density adjusted to 10^7 CFU/ml for bacteria and 10^6 CFU/ml for yeast. Cells were then treated with the MIC of peptides and incubated for two hours at 37 °C for bacteria and 28 °C for yeast, respectively. As the MIC of peptides was determined with lower number of cells, microbes are essentially treated with sub-MIC concentrations for FESEM analysis. This ensures that the cells are not completely lysed and microbes with small defects can also be observed. Cell suspensions were then centrifuged at 1000 RCF for 5 min followed by treatment with 2.5% glutaraldehyde and incubated at 4 °C for 3 h. Cells were washed twice with 10 mM phosphate buffer, pH 7.4 and loaded on glass slides. Air dried cells were then washed with deionized water followed by a gradient of alcohol ranging from 30 to 100%. Finally, the cells were air-dried, coated with gold, and FESEM images recorded.

3. Results

3.1. The peptides are predicted to be membrane-seeking ones

The C-terminal region of FtsA folds into an amphipathic helix that anchors the protein to membrane [18]. The peptides synthesized for antimicrobial studies are shown in Table 1. Mean hydrophobicity (< H >) and mean hydrophobic helical moments (< μH >) were calculated using Heliquest web server [22]. The discrimination factor, D is calculated using formula, D = 0.944 < μH > + 0.33(z) [22,23]. As discrimination factor is larger than 1.34, all the peptides are predicted to be membrane-binding ones. Comparison of the hydrophobicity and hydrophobic moments with some known α-helical antimicrobial peptides is shown in Fig. S1 and Table S1 (Supplementary data).

Table 1
Sequences and physicochemical properties of the peptides.

Peptide	Peptide sequence[a]	z[b]	< H >	< μH >	D
FtsA10	IKRLNSWLRK-am	+5	0.281	0.761	2.37
Ac-FtsA10	Ac-IKRLNSWLRK-am	+4	0.281	0.761	2.04
FtsA11	WIKRLNSWLRK-am	+5	0.460	0.843	2.45
Ac-FtsA11	Ac-WIKRLNSWLRK-am	+4	0.460	0.843	2.12
FtsA13	GSWIKRLNSWLRK-am	+5	0.386	0.716	2.33
Ac-FtsA13	Ac-GSWIKRLNSWLRK-am	+4	0.386	0.716	2.00

[a] 'Ac-' at N-terminus indicates amino-terminal acetylation, while '-am' at C-terminus indicates C-terminal amide.
[b] Net charge on the peptide at pH 7.4.

3.2. The peptides exhibit preferential binding to negatively-charged lipid vesicles

Binding of the peptides with lipid vesicles was studied using steady-state tryptophan fluorescence. Fluorescence emission spectra were examined in HEPES-buffered saline in the absence of lipid vesicles and in the presence of POPC:CHL (10:1) and POPE:POPG (7:3) SUVs at peptide to lipid ratios of 1:50 and 1:100 (Fig. 1).

In HEPES-buffered saline, all the peptides except Ac-FtsA10 display emission maximum ~353 nm; Ac-FtsA10 displays emission maximum ~363 nm. In the presence of POPC:CHL SUVs, FtsA10 and Ac-FtsA10 display no appreciable change in the emission spectra. In the presence of POPE:POPG vesicles, however, distinct blue shift with enhancement in fluorescence intensity is observed suggesting binding to the lipid vesicles. FtsA11 shows small (≤5 nm) blue shift in the presence of POPC:CHL vesicles but a large blue shift (~15 nm) is observed in the presence of POPE:POPG vesicles. The data indicate preferential binding to the negatively-charged vesicles. Ac-FtsA11, FtsA13, and Ac-FtsA13 also display preferential binding to negatively-charged SUVs; there is, however, appreciable binding to POPC:CHL SUVs as well. Accessibility of tryptophan side-chains to solvent was assessed using dynamic quencher acrylamide. The Stern-Volmer plots for all the peptides were fit linearly (Fig. 2).

The K_{SV} value obtained from Stern-Volmer plot is an indicator of tryptophan accessibility to quencher; high K_{SV} value indicates higher accessibility. The K_{SV} values of the peptides decrease in the presence of lipid vesicles suggesting membrane binding. The tryptophan is better shielded from quenching in the presence of negatively-charged vesicles. Net accessibility factor (NAF) was determined using the formula: NAF = K_{SV} (with SUVs)/K_{SV} (without SUVs) and is shown in Table 2. The extent of quenching is lesser in the presence of POPE:POPG vesicles compared to that in the presence of POPC:CHL vesicles.

3.3. The peptides combat Gram-negative, Gram-positive bacteria, and yeast

Activity of the peptides was tested against *E. coli*, a Gram-negative bacterium; gentamicin-methicillin-resistant *S. aureus* (gentamicin-resistant MRSA), a Gram-positive bacterium, and *C. albicans*, an opportunistic fungus. MIC values against these microbes are shown in Table 3.

The peptides, by and large, display better activity against *E. coli* followed by *C. albicans* and gentamicin-resistant MRSA. FtsA10 display an MIC of 64 μM against *E. coli* and *C. albicans*. N-terminal acetylation of the peptide improved the activity against *E. coli* (MIC = 32 μM). Both the 10-residue peptides, however, failed to inhibit the growth of gentamicin-resistant MRSA even at 128 μM concentration. The 11 and 13-residue peptides, on the other hand, exhibited activity against all three microorganisms. All the four peptides displayed strong activity against *E. coli* with MIC values ≤8 μM. Against *C. albicans*, FtsA11 displayed an MIC of 16 μM; the other three peptides displayed MIC ≤8 μM. The activity against gentamicin-resistant MRSA, however, was much lower; FtsA11 displayed an MIC of 64 μM whereas other three peptides displayed MIC of 32 μM. The cell wall of Gram-positive bacteria contains lipoteichoic acids whereas Gram-negative bacteria contain lipopolysaccharide (LPS) as a major component of their outer membrane. These anionic molecules are bridged by the divalent cations *viz.* Ca^{2+} and Mg^{2+} [24]. Growth media deficient in these cations might compromise bacterial cell-wall integrity thereby affecting their susceptibility to AMPs. As binding of AMPs to negatively-charged bacterial surface is mediated through electrostatic interaction, presence of salt can interfere with this binding. Diminished activity in the presence of salt and divalent cations has indeed been reported for many AMPs [25–29]. It is therefore imperative to test the activity of the peptides in the presence of salts and divalent cations at physiologically-relevant concentrations. The sensitivity of peptides to salt and divalent cations was, therefore assessed by determining the MIC values in the presence of 100 mM

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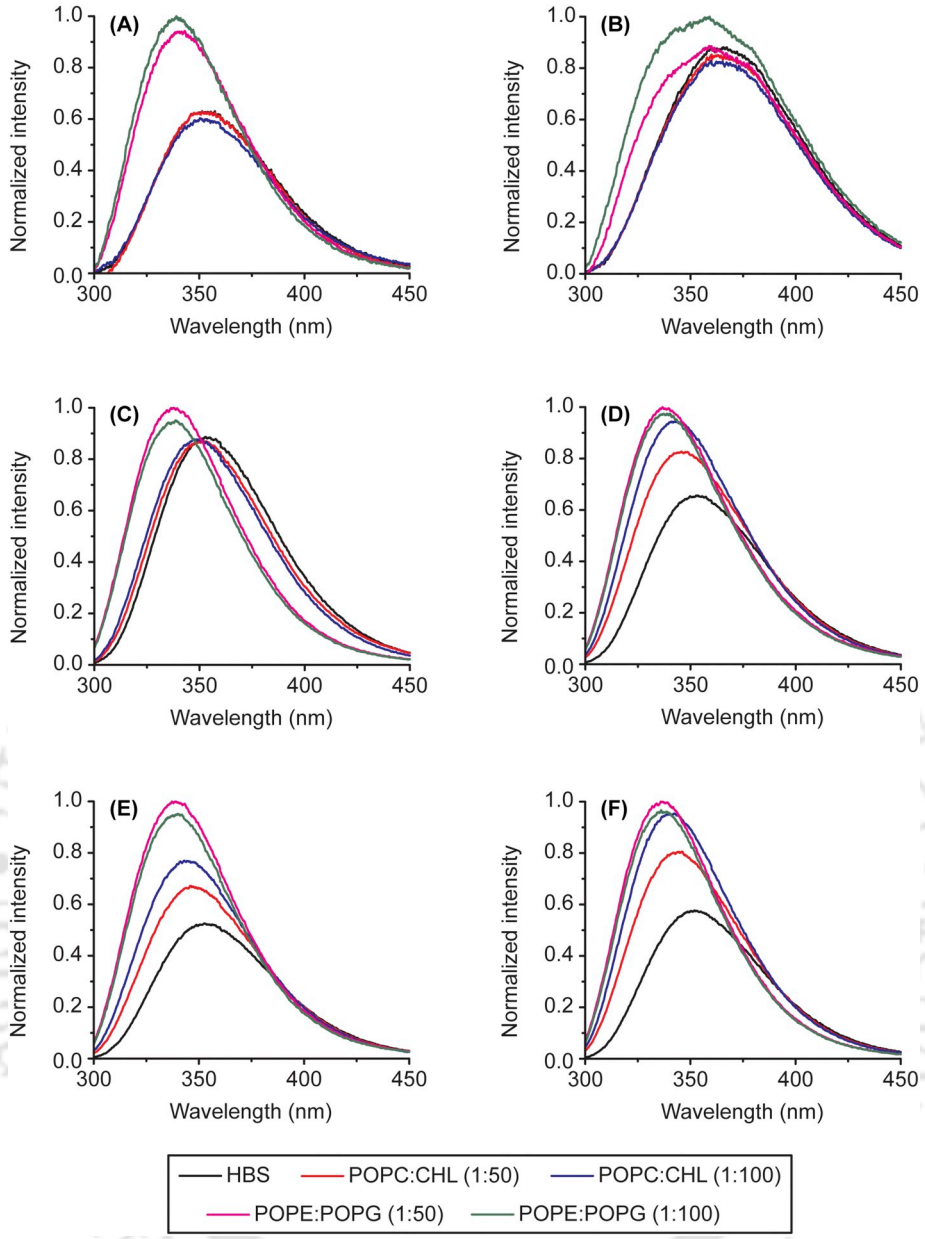

Fig. 1. Tryptophan fluorescence emission spectra of the FtsA peptides. (A) FtsA10, (B) Ac-FtsA10, (C) FtsA11, (D) Ac-FtsA11, (E) FtsA13, and (F) Ac-FtsA13.

NaCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$. The data are shown in Table 4.

The peptides, in general, displayed higher MIC values (lower activity) in the presence of salt and divalent cations. However, it is interesting to note that the MIC values of 11- and 13-residue peptides against *E. coli* are ≤8 μM under these conditions as well. Interestingly, FtsA11 and Ac-FtsA13 displayed same MIC values against *E. coli* under all the conditions tested. MIC of Ac-FtsA11 and FtsA13 against gentamicin-resistant MRSA is same in the presence of salt and divalent cations (32 μM). Against *C. albicans*, FtsA13 turns out to be the most potent peptide; the peptide displays an MIC of 16 μM in the presence of $CaCl_2$ while lower MICs were obtained in the presence of NaCl and $MgCl_2$. None of the peptides caused > 1.5% hemolysis up to 32 μM concentration (Table S2). At a four-fold higher concentration of 128 μM, however, Ac-FtsA11, FtsA13, and Ac-FtsA13 caused ~1.2%, ~4.2%, and ~3.6% hemolysis, respectively. These data indicate that the 11- and 13-residue peptides are promising antimicrobial candidates. Interestingly though, the peptides display selectivity towards *E. coli* than *S. aureus*. To investigate if the specificity is towards *E. coli* or Gram-negative bacteria, in general, the peptides were tested against *S. enterica* and *P. aeruginosa* as well. The 11- and 13-residue peptides efficiently killed the microbes at MIC ≤16 μM (Table S3). The data indicate that the peptides are more selective for Gram-negative bacteria than Gram-positive bacteria. FESEM imaging of the peptide-treated microbes indicate abnormal cell morphology compared to the untreated microbes (Fig. 3). These data indicate membrane permeabilization as the possible mechanism of peptide action that is validated by outer and inner membrane permeabilization of *E. coli*. Other than Ac-FtsA13, all the peptides cause large-scale disruption of bacteria. Ac-FtsA13, on the other hand, resulted in blebs on bacterial surface. Interestingly, the peptide causes ~50% outer-membrane permeabilization at its MIC. This indicates that membrane permeabilization alone may not be responsible for the killing.

3.4. The peptides exhibit potent outer-membrane-permeabilizing activity with E. coli cells

Permeabilization of *E. coli* outer membrane was investigated using NPN fluorometric assay. NPN is a fluorescent dye that exhibits

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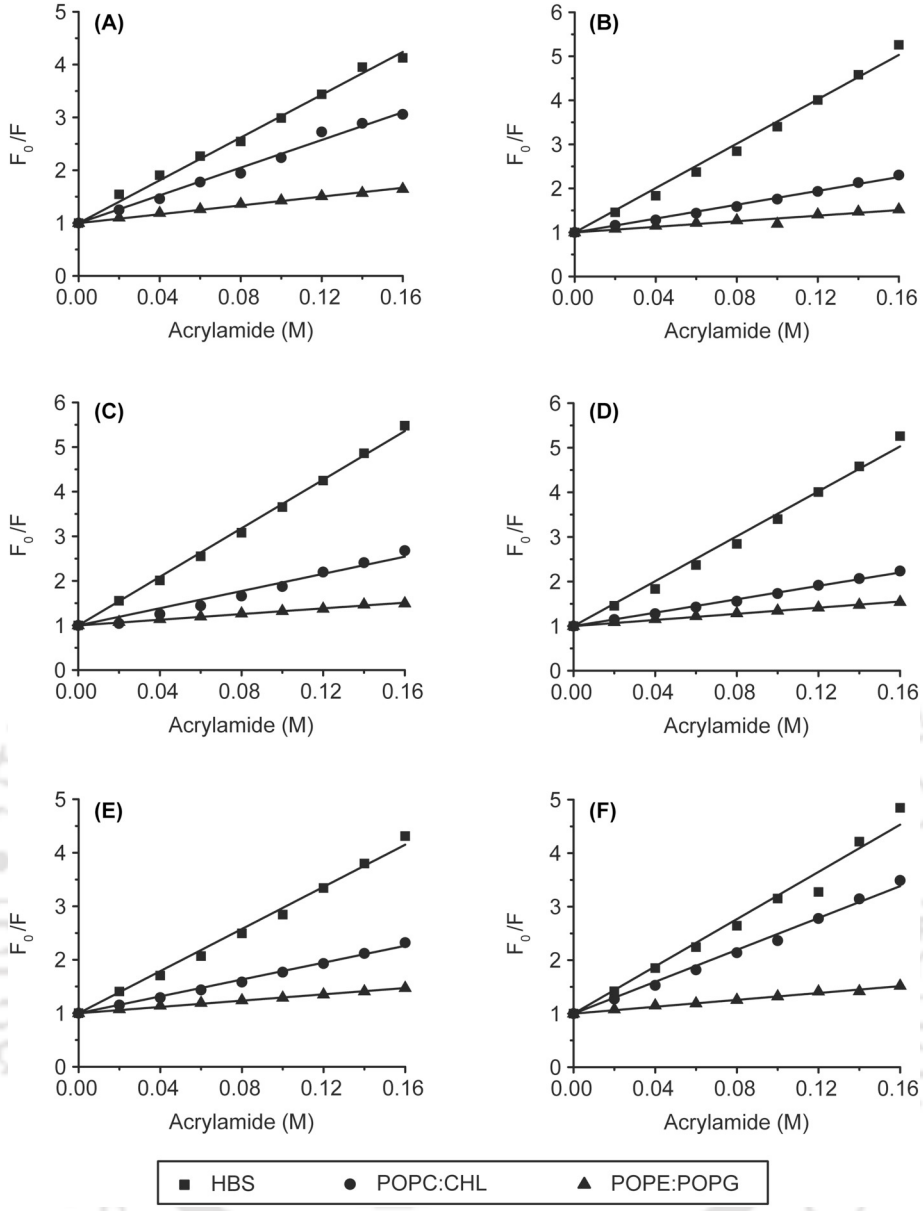

Fig. 2. Stern Volmer plots for tryptophan quenching by acrylamide. Where, (A) FtsA10, (B) Ac-FtsA10, (C) FtsA11, (D) Ac-FtsA11, (E) FtsA13, and (F) Ac-FtsA13. F_0 is the tryptophan fluorescence intensity at 350 nm in the absence of quencher whereas F is the fluorescence intensity in the presence of quencher.

Table 2
K_{sv} and NAF values for the FtsA-derived peptides.

Peptide	HBS		POPC:CHL		POPE:POPG	
	K_{sv} (M^{-1})	NAF	K_{sv} (M^{-1})	NAF	K_{sv} (M^{-1})	NAF
FtsA10	20.24	1	13.11	0.64	4.17	0.20
Ac-FtsA10	25.19	1	7.86	0.31	3.17	0.12
FtsA11	27.24	1	9.64	0.35	3.19	0.11
Ac-FtsA11	25.19	1	7.52	0.29	3.44	0.13
FtsA13	19.68	1	7.88	0.40	2.94	0.14
Ac-FtsA13	22.07	1	14.89	0.67	3.21	0.14

Table 3
MIC of the FtsA-derived peptides.

Peptide	Minimum inhibitory concentration (μM)		
	E. coli	Gentamicin-resistant MRSA	*C. albicans*
FtsA10	64	> 128	64
Ac-FtsA10	32	> 128	64
FtsA11	8	64	16
Ac-FtsA11	2	32	8
FtsA13	2	32	4
Ac-FtsA13	4	32	8

enhanced fluorescence in hydrophobic environment. An enhancement in NPN fluorescence intensity on peptide addition indicates perturbation of outer *E. coli* membrane. Polymyxin B, an antimicrobial peptide, is a well known outer-membrane-permeabilizing agent and is often used as a reference to compare the outer membrane permeabilization of novel compounds. Table 5 shows the percentage NPN uptake by *E. coli* cells at different peptide concentrations. NPN uptake on treatment with 10 μg/ml polymyxin B was taken to be 100%.

All the peptides exhibit > 40% uptake at 2 μM concentration; FtsA13 displayed 100% uptake. FtsA10 and Ac-FtsA10 resulted in 100% uptake at 4 μM concentration (Table 5). The other three peptides did not result in 100% uptake up to 8 μM concentration; Ac-FtsA11 and Ac-

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Table 4
MIC of the FtsA-derived peptides in the presence of salt and divalent cations.

Peptides	Minimum inhibitory concentration (μM)								
	E. coli			Gentamicin-resistant MRSA			*C. albicans*		
	NaCl	$CaCl_2$	$MgCl_2$	NaCl	$CaCl_2$	$MgCl_2$	NaCl	$CaCl_2$	$MgCl_2$
FtsA10	128	128	64	> 128	> 128	> 128	> 128	> 128	128
Ac-FtsA10	64	64	32	> 128	> 128	> 128	128	> 128	64
FtsA11	8	8	8	128	128	128	32	64	16
Ac-FtsA11	8	4	4	32	32	32	16	32	16
FtsA13	8	4	2	32	32	32	8	16	4
Ac-FtsA13	8	8	8	32	64	64	8	32	8

FtsA13, however, displayed ~98% uptake. FtsA11 caused similar NPN uptake (~60–70%) at all the three concentrations tested. It is interesting to note that FtsA10 and Ac-FtsA10 display 100% uptake at 4 μM concentration whereas they display MIC values of 64 μM and 32 μM respectively. Contrarily, FtsA11, Ac-FtsA11, Ac-FtsA13 display ~71%, 83%, and 51% NPN uptake at their MIC. The data indicate that outer-membrane permeabilization alone cannot be correlated to the antimicrobial activity. Hancock and coworkers have observed similar lack of correlation between membrane permeabilization and MIC of the peptides [25,30].

3.5. The peptides cause rapid permeabilization of *E. coli* inner-membrane

The voltage-sensitive dye $diSC_3(5)$ was used to determine the depolarization of *E. coli* inner-membrane. $DiSC_3(5)$ partitions to the polarized membranes, gets concentrated, and self-quenched. Dissipation of membrane potential causes release of dye into the medium thereby resulting in enhanced fluorescence. Polymyxin B was used as the positive control for membrane permeabilization. Three independent experiments were carried out and the representative data are shown in Fig. 4.

All the peptides other than FtsA10 caused gradual increase in $diSC_3(5)$ fluorescence intensity indicating inner-membrane permeabilization (Fig. 4). Despite being an efficient outer-membrane-permeabilizing agent, FtsA10 failed to cause a noticeable increase in $diSC_3(5)$ fluorescence. The differential behavior could possibly arise due to peptide's binding strength to the outer membrane. The peptide possesses high charge density compared to the other peptides and the charge is concentrated near the peptide termini, which could influence the way the peptide interacts with the outer-membrane. A strong binding to outer-membrane lipids could limit the peptide's access to inner-membrane. Ac-FtsA10 shows gradual increase in the fluorescence intensity indicating permeabilization of inner-membrane (panel B). Similar enhancement is observed for FtsA11 (panel C), Ac-FtsA11 (panel D), FtsA13 (panel E), and Ac-FtsA13 (panel F). Polymyxin B causes similar enhancement in $diSC_3(5)$ fluorescence (panel G) indicating that the peptides are as effective membrane-permeabilizing agents as polymyxin B. Bacterial cells do not show any change in the $diSC_3(5)$ fluorescence on the experimental time-scale (negative control, panel H). These data suggest membrane permeabilization by the peptides as the mechanism of antimicrobial action.

4. Discussion

The golden era (1950s–1970s) of antibiotics could arguably be called the golden era in medicine. More than half of the antibiotics that are in use even today were discovered in this period. Post that era, the discovery of new antibiotics has failed to match the rate of emergence of antibiotic-resistant bacteria. The seriousness of the threat could be realized from the fact that microbial resistance spans all known antibiotic classes. The second golden age of antibiotics is eagerly awaited in healthcare. Largely due to their use as defense molecule across the biological kingdoms and their direct mechanism of killing, peptides turn out to be one of the most promising candidates as antimicrobials. AMPs are ancient defense molecules, but resistance against them is not prevalent. In fact, mutations in the microbes to counter AMPs has resulted in their diversification. A majority of these peptides are cationic in nature and permeabilize bacterial membranes by adopting an amphipathic structure. We reasoned that the membrane-binding peptide stretches in bacterial proteins could be purposed into potent membrane-perturbing antimicrobials. The peptides derived from the C-terminal amphipathic helix of *E. coli* FtsA protein were predicted to be membrane-seeking ones by HeliQuest [22]. The peptides exhibit preferential binding to the negatively-charged lipid vesicles as indicated by Trp fluorescence studies. Antimicrobial assays against *E. coli, S. enterica, and P. aeruginosa* (Gram-negative); gentamicin-resistant MRSA (Gram-positive); and *C. albicans* (yeast) gave promising results for the 11- and 13-residue peptides. All the four peptides show MIC values ≤8 μM against *E. coli* and *S. enterica*. *P. aeruginosa* was also killed by Ac-FtsA11, FtsA13, and Ac-FtsA13 at 8 μM concentration; FtsA11, on the other hand, displayed an MIC of 16 μM. MIC values against gentamicin-resistant MRSA turned out to be at least 4-fold higher indicating selectivity towards Gram-negative bacteria. This is quite intriguing as Gram-positive bacteria, in general, have higher content of negatively-charged lipids and most AMPs reported in the literature show better activity against Gram-positive bacteria. We used gentamicin-resistance MRSA in this study as the model Gram-positive bacterium. *S. aureus* membrane, however, can have very high content of cationic lipid, lysylphosphatidylglycerol thereby diminishing the overall charge of the membrane thereby countering the AMPs. The *E. coli* FtsA-derived peptides used in this study are highly amphipathic but only moderately hydrophobic; less hydrophobic peptides could display selectivity towards Gram-negative bacteria [16,30]. Furthermore, it is likely that membrane permeabilization is not the sole mechanism of killing, the peptides may interfere with the intracellular molecules once they permeabilize the cytoplasmic membrane of the bacteria. The peptide stretch in *S. aureus* FtsA protein homologous to the 13-residue stretch of *E. coli* FtsA is quite different in sequence. Furthermore, unlike *E. coli* FtsA, the peptide stretch does not lie near the protein terminus; the stretch is followed by 50-residues. It would be interesting to study if the *S. aureus* fragment would show better activity against *S. aureus*. The 11- and 13-residue AMPs could inhibit the opportunistic fungus, *C. albicans* as well at promising MIC ≤16 μM.

The disinterest of most pharmaceutical companies in identifying new antibiotics is upsetting [31]. The slow discovery of new antibiotics make drug-resistant microbes a serious health threat. The threat is particularly high from Gram-negative bacteria as all the new classes of antibiotics that entered medicine in past five decades are all against Gram-positive bacteria [32]. The recently discovered antibiotics, teixobactin and malacidins are also active against Gram-positive bacteria [33,34]. Gram-negative bacteria have become increasingly drug-resistant; some species possess intrinsic resistance against antibiotics and others can easily acquire the trait through horizontal gene transfer. It is interesting to find that *E. coli*-derived peptides (11 and 13-residue FtsA-

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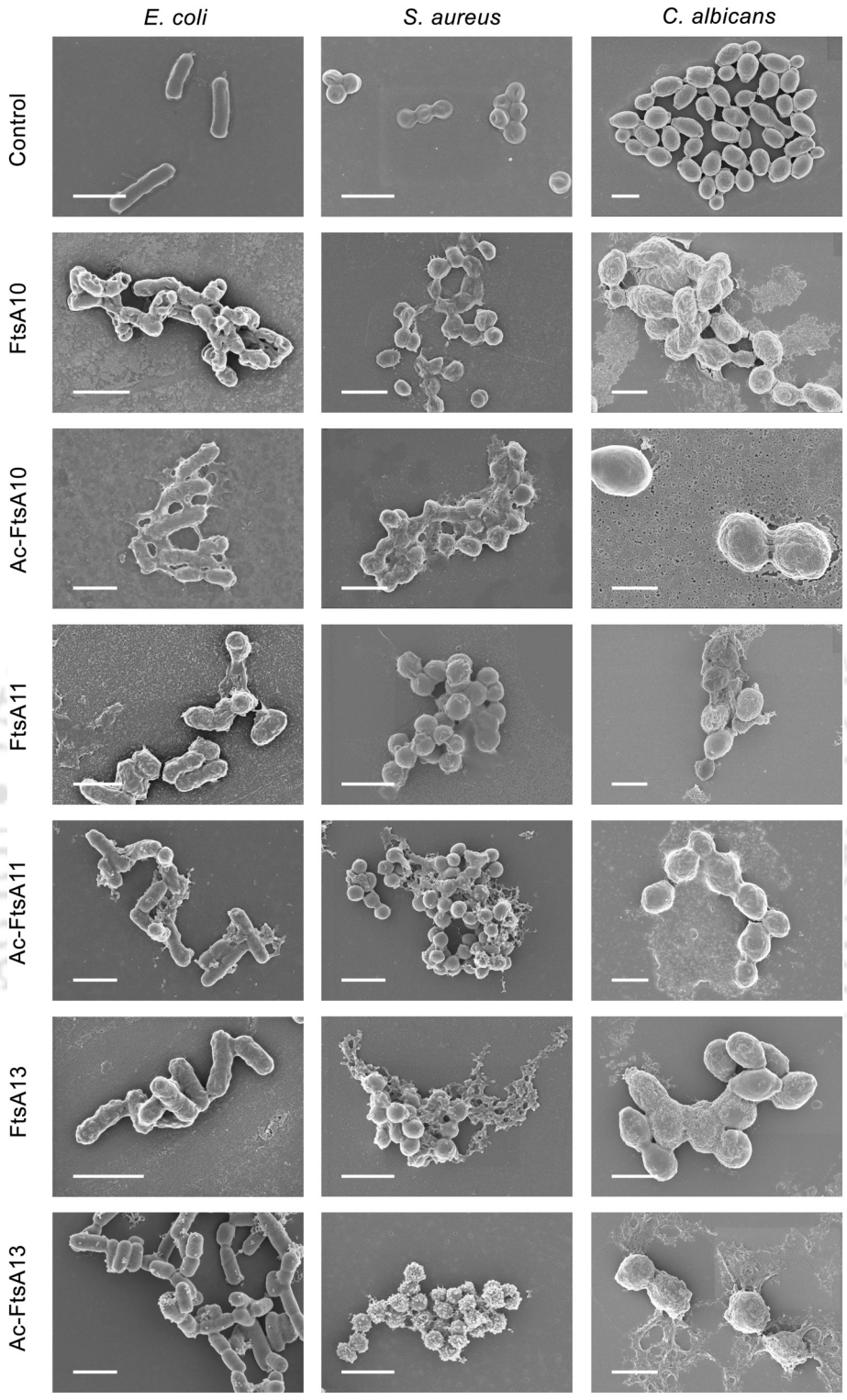

Fig. 3. FESM micrographs of the peptide-treated microbes. The cells were treated with peptides as discussed in the 'Material and methods' section and analyzed using FESEM. The scale bars represent 2 μm.

derived peptides) could combat the Gram-negative bacteria efficiently, even in the presence of salt and divalent cations. The membrane permeabilization assays with *E. coli* indicate outer- and inner-membrane permeabilization as the mechanism of killing, much like polymyxins that are used to treat drug-resistant Gram-negative bacteria nosocomial infections [35]. Other modes of killing, however, are very much possible. Once inside the bacterial cell, the FtsA-derived peptides might interfere with the physiological functions of the native FtsA protein.

AMPs derived from membrane-binding stretches of pathogenic organism's proteins could be an interesting antimicrobial strategy. The peptides could kill the very same microbe by permeabilizing the membrane and possibly by disturbing the homeostasis if they bind to the intracellular targets of the parent protein. In addition to that, the peptides could combat other microbes as well through usual membrane permeabilization mechanism as is observed for *E. coli* FtsA-derived peptides; the peptides could inhibit both gentamicin-resistant MRSA

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Table 5
Outer-membrane permeabilization of *E. coli* cells by FtsA-derived peptides. Percentage NPN uptake was determined relative to that caused by 10 μg/ml polymyxin B. The numbers in parentheses indicate the MIC of the peptides against *E. coli*.

Peptide concentration	FtsA10 (64 μM)	Ac-FtsA10 (32 μM)	FtsA11 (8 μM)	Ac-FtsA11 (2 μM)	FtsA13 (2 μM)	Ac-FtsA13 (4 μM)
2 μM	49.76 ± 22.62	87.3 ± 7.84	63.5 ± 6.9	82.95 ± 18.99	100	43.76 ± 12.33
4 μM	100	100	68.8 ± 12.14	85.56 ± 14	100	50.84 ± 17.85
8 μM	–	–	71.2 ± 15.06	98.40 ± 2.25	–	97.79 ± 3.12

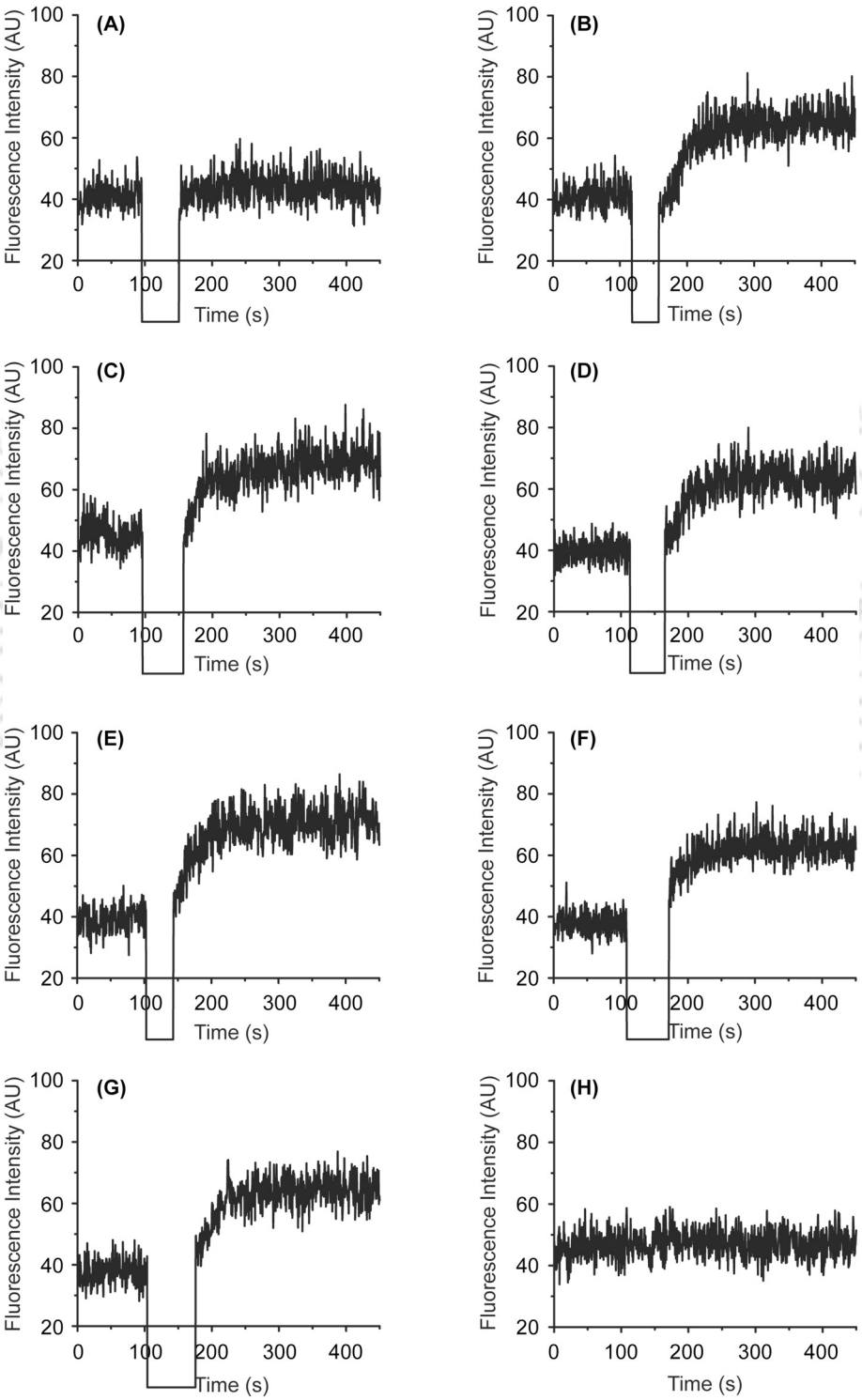

Fig. 4. Inner membrane depolarization assay performed on *E. coli* cells. (A) FtsA10, (B) Ac-FtsA10, (C) FtsA11, (D) Ac-FtsA11, (E) FtsA13, (F) Ac-FtsA13, (G) Polymyxin B, (H) Cells without peptide.

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and *C. albicans*, albeit at higher concentrations. Development of resistance against membrane-targeting molecules is less common. Moreover, it would be interesting to test if self-like peptide sequences can escape the proteolytic cleavage by the bacteria.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

NC conceived the idea. KS and NC designed the experiments. KS performed the experiments and analyzed the results. KS wrote the manuscript with NC.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbamem.2018.09.011.

References

[1] M. Mahlapuu, J. Hakansson, L. Ringstad, C. Bjorn, Antimicrobial peptides: an emerging category of therapeutic agents, Front. Cell. Infect. Microbiol. 6 (2016) 194.
[2] R.E. Hancock, H.G. Sahl, Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies, Nat. Biotechnol. 24 (12) (2006) 1551–1557.
[3] K.A. Brogden, Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat. Rev. Microbiol. 3 (3) (2005) 238–250.
[4] P. Bulet, R. Stocklin, L. Menin, Anti-microbial peptides: from invertebrates to vertebrates, Immunol. Rev. 198 (2004) 169–184.
[5] A. Tossi, L. Sandri, A. Giangaspero, Amphipathic, alpha-helical antimicrobial peptides, Biopolymers 55 (1) (2000) 4–30.
[6] I. Zelezetsky, A. Tossi, Alpha-helical antimicrobial peptides–sing a sequence template to guide structure-activity relationship studies, Biochim. Biophys. Acta 1758 (9) (2006) 1436–1449.
[7] N. Sitaram, R. Nagaraj, Interaction of antimicrobial peptides with biological and model membranes: structural and charge requirements for activity, Biochim. Biophys. Acta 1462 (1–2) (1999) 29–54.
[8] Y. Shai, Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by alpha-helical antimicrobial and cell non-selective membrane-lytic peptides, Biochim. Biophys. Acta 1462 (1–2) (1999) 55–70.
[9] K.J. Hallock, D.K. Lee, A. Ramamoorthy, MSI-78, an analogue of the magainin antimicrobial peptides, disrupts lipid bilayer structure via positive curvature strain, Biophys. J. 84 (5) (2003) 3052–3060.
[10] C. Monteiro, M. Pinheiro, M. Fernandes, S. Maia, C.L. Seabra, F. Ferreira-Da-Silva, S. Reis, P. Gomes, M.C. Martins, A 17-mer membrane-active MSI-78 derivative with improved selectivity toward bacterial cells, Mol. Pharm. 12 (8) (2015) 2904–2911.
[11] I.Y. Park, J.H. Cho, K.S. Kim, Y.B. Kim, M.S. Kim, S.C. Kim, Helix stability confers salt resistance upon helical antimicrobial peptides, J. Biol. Chem. 279 (14) (2004) 13896–13901.
[12] H.L. Chu, H.Y. Yu, B.S. Yip, Y.H. Chih, C.W. Liang, H.T. Cheng, J.W. Cheng, Boosting salt resistance of short antimicrobial peptides, Antimicrob. Agents Chemother. 57 (8) (2013) 4050–4052.
[13] P.K. Hazam, G. Jerath, A. Kumar, N. Chaudhary, V. Ramakrishnan, Effect of tacticity-derived topological constraints in bactericidal peptides, Biochim. Biophys. Acta Biomembr. 1859 (8) (2017) 1388–1395.
[14] K. Saikia, Y.D. Sravani, V. Ramakrishnan, N. Chaudhary, Highly potent antimicrobial peptides from N-terminal membrane-binding region of *E. coli* MreB, Sci. Rep. 7 (2017) 42994.
[15] K. Saikia, N. Chaudhary, Interaction of MreB-derived antimicrobial peptides with membranes, Biochem. Biophys. Res. Commun. 498 (1) (2018) 58–63.
[16] N. Malanovic, K. Lohner, Antimicrobial peptides targeting gram-positive bacteria, Pharmaceuticals 9 (3) (2016).
[17] J.R. Herricks, D. Nguyen, W. Margolin, A thermosensitive defect in the ATP binding pocket of FtsA can be suppressed by allosteric changes in the dimer interface, Mol. Microbiol. 94 (3) (2014) 713–727.
[18] S. Pichoff, J. Lutkenhaus, Tethering the Z ring to the membrane through a conserved membrane targeting sequence in FtsA, Mol. Microbiol. 55 (6) (2005) 1722–1734.
[19] S. Pichoff, B. Shen, B. Sullivan, J. Lutkenhaus, FtsA mutants impaired for self-interaction bypass ZipA suggesting a model in which FtsA's self-interaction competes with its ability to recruit downstream division proteins, Mol. Microbiol. 83 (1) (2012) 151–167.
[20] I. Wiegand, K. Hilpert, R.E. Hancock, Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances, Nat. Protoc. 3 (2) (2008) 163–175.
[21] L. Zhang, P. Dhillon, H. Yan, S. Farmer, R.E. Hancock, Interactions of bacterial cationic peptide antibiotics with outer and cytoplasmic membranes of *Pseudomonas aeruginosa*, Antimicrob. Agents Chemother. 44 (12) (2000) 3317–3321.
[22] R. Gautier, D. Douguet, B. Antonny, G. Drin, HELIQUEST: a web server to screen sequences with specific alpha-helical properties, Bioinformatics 24 (18) (2008) 2101–2102.
[23] R.C. Keller, The prediction of novel multiple lipid-binding regions in protein translocation motor proteins: a possible general feature, Cell. Mol. Biol. Lett. 16 (1) (2011) 40–54.
[24] M. Zasloff, Antimicrobial peptides of multicellular organisms, Nature 415 (6870) (2002) 389–395.
[25] M. Wu, E. Maier, R. Benz, R.E. Hancock, Mechanism of interaction of different classes of cationic antimicrobial peptides with planar bilayers and with the cytoplasmic membrane of *Escherichia coli*, Biochemistry 38 (22) (1999) 7235–7242.
[26] M.J. Goldman, G.M. Anderson, E.D. Stolzenberg, U.P. Kari, M. Zasloff, J.M. Wilson, Human β-Defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis, Cell 88 (4) (1997) 553–560.
[27] J.Y. Lee, S.-T. Yang, S.K. Lee, H.H. Jung, S.Y. Shin, K.-S. Hahm, J.I. Kim, Salt-resistant homodimeric bactenecin, a cathelicidin-derived antimicrobial peptide, FEBS J. 275 (15) (2008) 3911–3920.
[28] J. Varkey, R. Nagaraj, Antibacterial activity of human neutrophil defensin HNP-1 analogs without cysteines, Antimicrob. Agents Chemother. 49 (11) (2005) 4561–4566.
[29] J. Varkey, S. Singh, R. Nagaraj, Antibacterial activity of linear peptides spanning the carboxy-terminal beta-sheet domain of arthropod defensins, Peptides 27 (11) (2006) 2614–2623.
[30] C.L. Friedrich, D. Moyles, T.J. Beveridge, R.E.W. Hancock, Antibacterial action of structurally diverse cationic peptides on gram-positive bacteria, Antimicrob. Agents Chemother. 44 (8) (2000) 2086–2092.
[31] M.L. Katz, L.V. Mueller, M. Polyakov, S.F. Weinstock, Where have all the antibiotic patents gone? Nat. Biotechnol. 24 (12) (2006) 1529–1531.
[32] A.K. Marr, W.J. Gooderham, R.E. Hancock, Antibacterial peptides for therapeutic use: obstacles and realistic outlook, Curr. Opin. Pharmacol. 6 (5) (2006) 468–472.
[33] L.L. Ling, T. Schneider, A.J. Peoples, A.L. Spoering, I. Engels, B.P. Conlon, A. Mueller, T.F. Schaberle, D.E. Hughes, S. Epstein, M. Jones, L. Lazarides, V.A. Steadman, D.R. Cohen, C.R. Felix, K.A. Fetterman, W.P. Millett, A.G. Nitti, A.M. Zullo, C. Chen, K. Lewis, A new antibiotic kills pathogens without detectable resistance, Nature 517 (7535) (2015) 455–459.
[34] B.M. Hover, S.H. Kim, M. Katz, Z. Charlop-Powers, J.G. Owen, M.A. Ternei, J. Maniko, A.B. Estrela, H. Molina, S. Park, D.S. Perlin, S.F. Brady, Culture-independent discovery of the malacidins as calcium-dependent antibiotics with activity against multidrug-resistant Gram-positive pathogens, Nat. Microbiol. 3 (4) (2018) 415–422.
[35] A.S. Michalopoulos, D.C. Karatza, Multidrug-resistant Gram-negative infections: the use of colistin, Expert Rev. Anti-Infect. Ther. 8 (9) (2010) 1009–1017.

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