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Prospecting Rumen Bacteria for Novel Antimicrobials

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Abstract

Due to their broad-spectrum and bactericidal activity, antimicrobial proteins and peptides (AMPPs) are considered as future drug alternatives to combat the escalating problem of antimicrobial resistance in medicine. The community of competitive culturable and non-culturable bacteria in the rumen present a potential source for the discovery of novel bioactive compounds including AMPPs.

Metagenomic and bioinformatics based techniques were used to prospect two rumen bacterial metagenomes for potentially novel antimicrobial genes (proteins) and peptide sequences. Novel short antimicrobial peptides (peptides 1-181) and eleven longer antimicrobial genes/miniproteins (Gene 6, 17A, 17B *palG*1 and *palG*2, H-G1, H-G2, H-G3, H-G4, H-G5) were identified. Eight of these (peptides 2, 3, 4, 5, 7, 8, 15 and *palG*1) were selected for further analysis. These antimicrobials displayed potent antimicrobial activity (minimum inhibitory concentrations ranging from 32 to 64 µg/ml) against both Gram positive bacteria strains (including Methicillin sensitive and resistant *Staphylococcus aureus* strains MSSA RN4220 and EMRSA-15, *Enterococcus faecalis* JH2-2 and *Listeria monocytogenes* NCTC 11994 (serovar 4b)), as well as Gram negative bacteria strains (*Escherichia coli* K12, *Salmonella enterica* serovar Typhimurium SL1344 and *Pseudomonas aeruginosa* (15692) PAO1 strain H103) in Mueller Hinton broth. No haemolytic activity against red blood cells was seen. Data obtained indicate that loss of cell viability is due to cytoplasmic leakage and there is some evidence of interference with the cell division mechanism.

The rumen AMPPs identified in this study show great activity against clinically relevant human pathogens and to our knowledge are the first rumen AMPs identified using metagenomics. Overall, the data support the potential use of AMPs (2, 3, 7), AMPs (2, 3, 4, 5, 7, 8, 15) and the polypeptide *palG*1 in the treatment of *S. aureus*, *E. coli* and *Ent. faecalis* infections respectively in the future.

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List of Abbreviations

1D	One-dimensional
3D	Three-dimensional
6-APA	6-aminopenicillanic acid
Å	Angstrom
AA	Amino acids
APD/APD2	Antimicrobial peptide database
AMB	Ammonium molybdate
AMPPs	Antimicrobial proteins and peptides
AMP	Antimicrobial peptide
ANOVA	Analysis of variance
AntiBP	Antibacterial peptides database
APS	Ammonium persulphate
Bac2A	Bactenecin
BPI	Bactericidal permeability-increasing protein
CAMP	Collection of Anti-Microbial Peptides
CD	Circular dichroism or Compact Disc
CFU	Colony forming units
CO_2	Carbondioxide
DA	Discriminant analysis
DAEC	Diffusely adherent *Escherichia coli*
DCM	Dichloromethane
DDR	D-alanyl-D-alanine residue
DES	DNase/ pyrogen-free water
DHFR	Dihydrofolate reductase
DIC	Diisoproplycabomide
diSC_3(5)	3,3′-dipropylthiadicarbocyanine iodide
DMF	Dimethlyformamide
DNA	Deoxyribonucleic acid
DPC	Dodecylphosphocholine
EAEC	Enteroaggregative *Escherichia coli*

EB	Elution buffer
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterohaemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
emPCR	Emulsion PCR
EPEC	Enteropathogenic *Escherichia coli*
ESBLs	Extended spectrum beta-lactamases
ETEC	Enterotoxigenic *Escherichia coli*
ExPEC	Extraintestinal pathogenic *Escherichia coli*
FDA	Food and Drug Administration Agency
Fmoc	Fluorenyl-methoxy-carbonyl
g	Gram(s)
H_2	Hydrogen
H_2O	Water
HBTU	O-(1H-Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate
HCAI	Healthcare associated infections
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H-G	Hess Gene
His-Tag	Histidine Tag
HLSCST	House of Lords Select Committee for Science and Technology
HMM-SA	Hidden Markov Model-derived structural alphabet
^{1}H NMR	Proton nuclear magnetic resonance
HOBt	Hydroxybenzotriazole
HPLC	High performance liquid chromatography
IBERS	Institute of Biological Environmental and Rural Sciences
IE	Infective endocarditis
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	Kilobases
kV	Kilovolts
LAB	Liquid associated bacteria
LAMP	A database linking antimicrobial peptides

LB	Luria-Bertani broth/agar
LPS	Lipopopolysaccharide
m	Meters
M	Molar
mA	Milliamperes
MATLAB	Matrix laboratory
MD	Molecular dynamics
MDR	Multidrug resistant
MG-RAST	Metagenomic Rapid Annotations using Subsystems Technology
MH	Mueller Hinton
MHA	Mueller Hinton Agar
MHB	Mueller Hinton Broth
MIC	Minimum inhibitory concentrations
MID	Multiplex identifiers
ml	Millilitres
mM	Millimolar
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-sensitive *Staphylococcus aureus*
NaOH	Sodium hydroxide
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
NCCLS	National Committee of Clinical Laboratory Standards
NCTC	National Collection of Type Cultures
ng	Nanogram
nm	Nanometres
NH_4^+	Ammonium
Ni-NTA	Nickel-nitrilotriacetic acid
nm	Nanomemeters
NMEC	Neonatal meningitis *Escherichia coli*
NMI	N-methylimidiazole
NMM	N-methylmorpholine
NMP	N-methyl-2-pyrrolidone

NMR	Nuclear magnetic resonance
OD	Optical density
OPEP	Optimal Potential for Efficient Peptide-Structure Prediction
ORF	Open reading frame
OTUs	Operation taxonomic units
PBP	Penicillin binding proteins
PCR	Polymerase chain reaction
PGN	Peptidoglycan
ρM	Picamole
PBS	Phosphate Buffered Saline
PPS	Protein Precipitation Solution
RAST	Rapid Annotations using Subsystems Technology
RBC	Red blood cells
rDNA	Ribosomal DNA
REMD	Replica Exchange Molecular Dynamics
RF	Random Forests
RMSD	Root-Mean-Square Deviation
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal RNA
SAB	Solid-attached bacteria
SAR	Structure activity relationships
SDS	Sodium dodecylsulfate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel
SFF	Standard Flowgram Format
SRF	Strained rumen fluid
ST	Heat-stable enterotoxin
STEC	Shiga toxin-producing *Escherichia coli*
SVM	Support vector machines
TAE	Tris-acetate-EDTA
TEMED	N, N, N‘, N‘, tetramethylenediamine
TFA	Trifluoroacetic acid

TIPS	Triisopropyl silane
Tm^{o}/ Tm	Melting Temperature
Tris-HCl	Tris-hydrochloride
TSST-1	Toxic shock syndrome toxin 1
UK	United Kingdom
µM	Micromolar
µl	Microlitres
UPEC	Uropathogenic *Escherichia coli*
USA	United States of America
UV	Ultraviolet light
V	Volt(s)
VFAs	Volatile fatty acids
VISA	Vancomycin intermediate *Staphylococcus aureus*
VRE	Vancomycin-resistant *Enterococci*
VRSA	Vancomycin-Resistant *Staphylococcus aureus*
WHO	World Health Organisation
~	Approximately
λ	Wavelength
α	Alpha

CHAPTER ONE

General Introduction

1.1 Introduction

The immediate post-World War 2 generation in developed countries was the first to enjoy, from birth, the benefits of a modern health system. These health systems largely abolished the pain of many infectious diseases, especially those of childhood, that ravaged societies in the past (Bennett 2008). This was made possible by the discovery and development of antibiotics in the 20th century, which marked a watershed in the treatment of very serious infections, stimulated advances in medical fields and enlarged the scope of medical care (Bennett 2008, Raghunath 2008).

Antibiotics may be natural, semi- or completely synthetic in origin. Although the term antibiotic was traditionally used to describe naturally-occurring substances produced by microorganisms (which in dilute solutions can selectively inhibit the growth of, or kill other microorganisms) and their derivatives, it is now more loosely used to describe both natural and synthetic agents (Scholar and Pratt 2000, Cooper et al. 2002). Since this discovery, a large number of antimicrobials belonging to different classes have been developed. These are distinct in their structural properties, modes of action, and spectra of organisms against which they are effective. Indeed, many of these have been extremely successful and have brought great benefits to human health (Chopra 2003, Projan and Ruzin 2006)

However, the intensive use and misuse of antibiotics, which was estimated in 2002 to be 100,000-200,000 tonnes per annum worldwide (Wise 2002, Andersson and Hughes 2010), has dramatically increased the frequency of antimicrobial resistance among human pathogens. This threatens a loss of therapeutic options, and a post-antibiotic era

in which present and future medical advances are threatened (Raghunath 2008, Andersson and Hughes 2010).

1.2 Antibiotic resistance

Antibiotic resistance is one of the greatest challenges for the twenty-first century (Rasko and Sperandio 2010). The global rise of antimicrobial resistance in bacteria, combined with the decreasing number of innovative antibacterial agents, has led to warnings that we may soon lose our ability to treat bacterial infections (WHO 2007). This is because the treatment of bacterial infections is increasingly complicated by microorganisms which develop resistance to antimicrobial agents (Martinez et al. 2007).

Antibiotic resistance in bacterial pathogens was identified very soon after the introduction of antibiotics into clinical practice as reviewed by (Mazel and Davies 1999). The prokaryotic cell is versatile and capable of adapting to the introduction of antibiotics into the environment. The inherent genetic variation provides a level of heterogeneity that ensures survivors in antibiotic charged environments (Raghunath 2008). A survey of bacterial isolates from the pre-antibiotic years show the presence of resistant organisms, although in small numbers (Medeiros 1997). However, in an antibiotic charged environment, a selection pressure builds up favouring the resistant organisms. This selective pressure which results in a steady rise in Minimum Inhibitory Concentrations (MICs) has led to resistance to all major antimicrobial classes (Lowy 2003, Rice 2006, Raghunath 2008) (see Table 1.1 for a timeline of antimicrobial discovery and resistance).

Table 1.1 Timeline of antibiotic discovery and resistance. Source (Lewis 2013)

Antibiotic class; example	Year of discovery	Year introduced	Year resistance observed	Mechanism of action	Activity or target species
Sulfadrugs; prontosil	1932	1936	1942	Inhibition of dihydropteroate synthetase	Gram-positive bacteria
β-lactams; penicillin	1928	1938	1945	Inhibition of cell wall biosynthesis	Broad-spectrum activity
Aminoglycosides; streptomycin	1943	1946	1946	Binding of 30S ribosomal subunit	Broad-spectrum activity
Chloramphenicols; chloramphenicol	1946	1948	1950	Binding of 50S ribosomal subunit	Broad-spectrum activity
Macrolides; erythromycin	1948	1951	1955	Binding of 50S ribosomal subunit	Broad-spectrum activity
Tetracyclines; chlortetracycline	1944	1952	1950	Binding of 30S ribosomal subunit	Broad-spectrum activity
Rifamycins; rifampicin	1957	1958	1962	Binding of RNA polymerase β-subunit	Gram-positive bacteria
Glycopeptides; vancomycin	1953	1958	1960	Inhibition of cell wall biosynthesis	Gram-positive bacteria
Quinolones; ciprofloxacin	1961	1968	1968	Inhibition of DNA synthesis	Broad-spectrum activity
Streptogramins; streptogramin B	1963	1998	1964	Binding of 50S ribosomal subunit	Gram-positive bacteria
Oxazolidinones; linezolid	1955	2000	2001	Binding of 50S ribosomal subunit	Gram-positive bacteria
Lipopetides; daptomycin	1986	2003	1987	Depolarization of cell membrane	Gram-positive bacteria
Fidaxomicin (targetingClostridium difficile)	1948	2011	1977	Inhibition of RNA polymerase	Gram-positive bacteria
Diarylquinolines; bedaquiline	1997	2012	2006	Inhibition of F1FO-ATPase	Narrow-spectrum activity (Mycobacterium tuberculosis)

Antimicrobial resistance is associated with increased patient morbidity and mortality rates, and has considerable economic consequences for healthcare systems (French 2005). For example, mortality rates in infections caused by Methicillin resistant *Staphylococcus aureus* (MRSA) are more than double those caused by Methicillin sensitive *S. aureus* (MSSA) (Whitby et al. 2001, Livermore 2003). Although there are still gaps in our knowledge, the overall significance of the problem of antibiotic resistance has subsequently led to an increased understanding of the contributing variables and molecular mechanisms underlying the development and spread of antimicrobial resistance, as evidenced by the large volume of literature on resistance.

There is therefore urgent need for the next generation of newer, safer and effective antibiotics and therapeutic treatment strategies, if we are to avoid a 'post-antibiotic' era and the negative societal and medical consequences of multidrug resistant bacterial infections (Alanis 2005).

1.3 Important pathogenic bacteria and current antibiotic treatment strategies

Over the past decade, there has been renewed public concern over infectious disease as a major public health threat. Human population growth over the years and an increasingly globalized microbial world has resulted in the emergence of infectious diseases (McMichael 2004). The spectrum of human pathogens and the infectious diseases they cause is continuously changing through evolution and changes in the way human populations interact with their environment and each other (Waldvogel 2004, van Doorn 2014). We are now faced with a growing population of pan-resistant bacteria that threaten to move us into a "postantibiotic era". Some of the more problematic drug-resistant pathogens encountered today include methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci* (VRE) and *Streptococcus pneumoniae*, among the Gram-positive bacteria and multidrug-resistant *Acinetobacter*

baumannii, *Klebsiella pneumoniae*, *Escherichia coli*, and *Pseudomonas aeruginosa* among the Gram-negative bacteria (Fish 2002, Lister et al. 2009). For the purpose of this thesis, MRSA, *E. coli* and *Enterococcus faecalis* are discussed below.

1.3.1 *Staphylococcus aureus*

S. aureus is a bacterium commonly found on the skin and in the nose of healthy people, its major habitats being the nasal membrane and the skin of warm-blooded animals (Whitt and Saylers 2002). *S. aureus* is a facultatively anaerobic, non-motile, Gram-positive coccus, around 0.6 μm in size, which appears as grape-like clusters when viewed through a microscope. It produces large, round, golden yellow colonies, often with haemolysis when grown on blood agar plates (Ryan and Ray 2004). *S. aureus* is catalase positive, that is, it is able to produce the enzyme catalase and convert hydrogen peroxide to water and oxygen. This makes the catalase test a very useful method to distinguish staphylococci from enterococci and streptococci. It is also primarily coagulase positive (i.e. produces an enzyme that causes clot formation (Ryan and Ray 2004).

S. aureus is recognised as the most frequently isolated nosocomial pathogen (Kuroda et al. 2001, Otto 2008). It can bring about complicated infections and serious hospital-acquired infections in all tissue types and organ systems due to its ability to produce an extensive array of cell surface proteins and secrete an abundance of extracellular enzymes like coagulase, protein A and toxins such as exfoliate toxins, superantigens, alpha, beta and delta toxins as well as toxic shock toxin (Projan and Norvick 1997, Lee and Bohach 2004), which promote disease by destroying constituents of host tissue, assisting dissemination of bacteria and counteracting host defence components (Ardvidson 2006, Lee and Lee. 2006) (Figure 1.1).

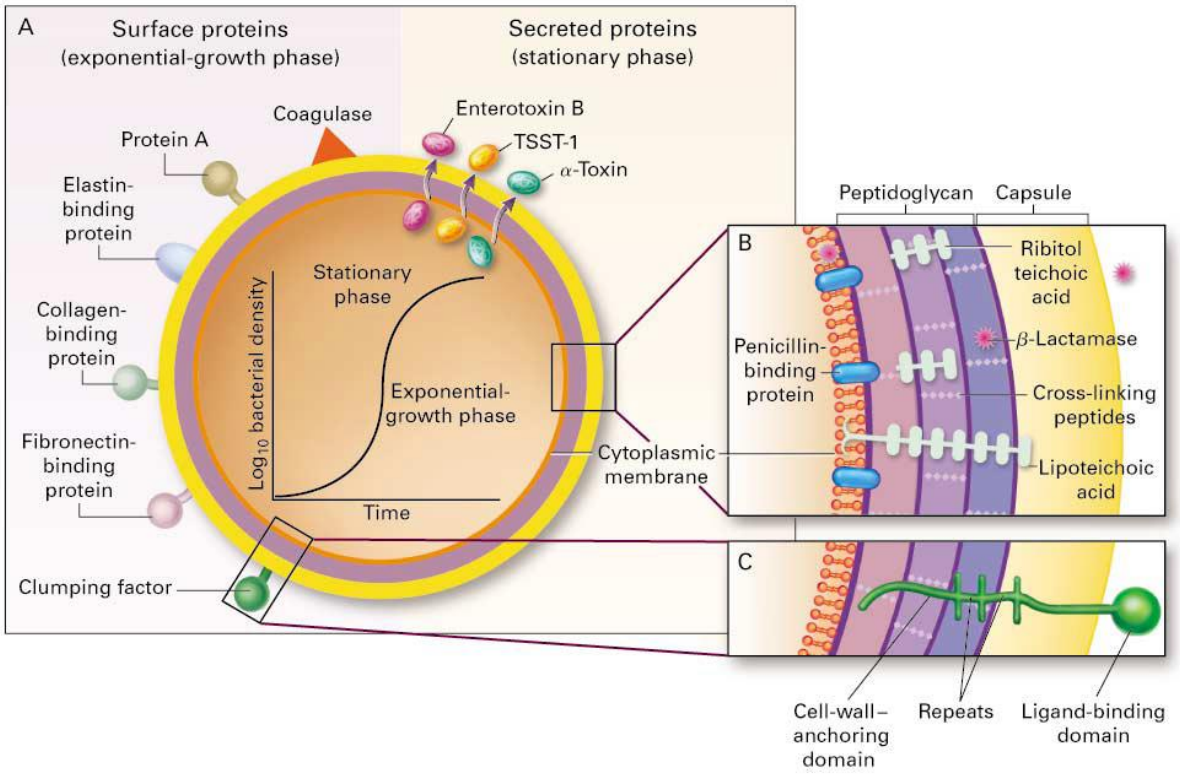


Figure 1.1 Structure of *S. aureus* and summary of its surface virulence factors. Panel A shows the surface and secreted proteins. The synthesis of many of these proteins is dependent on the growth phase, and is controlled by regulatory genes such as *agr*. Panels B and C show cross sections of the cell envelope. Many of the surface proteins have a structural organization similar to that of clumping factor, including repeated segments of amino acids (Panel C). TSST-1 denotes toxic shock syndrome toxin 1. Source: (Lowy 1998).

S. aureus readily acquires resistance against all classes of antibiotics by one of two distinct mechanisms: mutation of an existing bacterial gene or horizontal transfer of a resistance gene from another bacterium (Grundmann et al. 2006). Staphylococcal resistance to penicillin is mediated by penicillinase production (a form of β-lactamase); an enzyme which breaks down the β-lactam ring of the penicillin molecule (Lowy 2003, Kernodle 2006). Resistance problems encountered with the use of penicillin prompted the search for improved penicillin antibiotics (Bush 2003b). New β-lactams such as methicillin, oxacillin and flucloxacillin which were able to withstand penicillinase mediated hydrolysis and had anti-staphylococcal activity were derived from modification of the basic penicillin molecule (Greenwood 2003, Bush 2003a).

Methicillin-resistant *S. aureus* (MRSA) was identified in 1961, the same year Methicillin was introduced into clinical practice for the treatment of β-lactamase producing strains of staphylococci (Chopra 2003, Lowy 2003), which has spread rapidly to become recognized as a major threat to public health all over the world (Lindsay and Holden 2004). About 89 to 95% of *S. aureus* isolates have been found to be Methicillin resistant (Ippolito et al. 2010). This makes Methicillin-resistant *Staphylococcus aureus* (MRSA) the most commonly identified antibiotic-resistant pathogen in many parts of the world (Berger-Bachi and Rohrer 2002, Ug and Ceylan 2003, Grundmann et al. 2006). Methicillin resistance results from the expression of an additional penicillin binding protein (PBP), (PBP2') (through the acquisition of the *mecA* gene), which has a low affinity for β-lactams and is able to take over catalysis of essential transpeptidation reactions from the native PBPs inactivated by methicillin to maintain peptidoglycan cross-linkage (Chopra 2003). This renders the bacterial cell not only resistant to methicillin, but to most β-lactams (Hiramatsu et al. 1997, Davies et al. 2007, Stapleton et al. 2007)- see Figure 1.2 below for an illustration of the action of β-lactams on the cell.

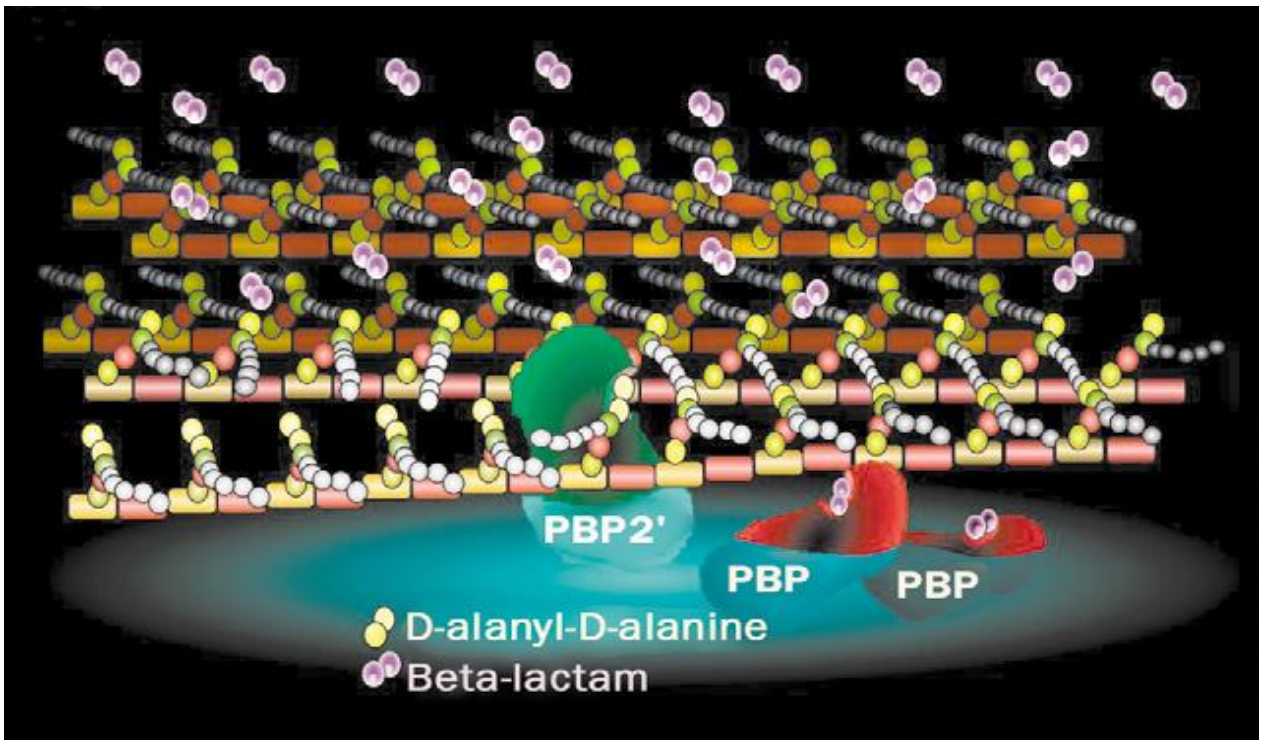


Figure 1.2 Action of beta-lactam antibiotics: the beta-lactam (purple double cubes) is a structural analogue of D-alanyl-D-alanine residues. It inactivates *S. aureus* PBPs (in red), but cannot bind to PBP2' (in green; MRSA-specific PBP) with high affinity. Therefore, MRSA can continue peptidoglycan synthesis in the presence of beta-lactams whereas methicillin-susceptible *S. aureus* cannot. Source: (Hiramatsu 2001).

As a result of the emergence of MRSA, glycopeptides (e.g. vancomycin and teicoplanin) naturally-occurring antibiotics with activity against many species of Gram-positive bacteria were introduced (Fraimow and Courvalin 2006). Vancomycin binds to the important substrates for the cell wall-synthesizing machinery, i.e., the D-alanyl-D-alanine residue (DDR) of the lipid II precursor, and thereby inhibits utilisation of the substrate by glycosyltransferases (cell wall synthesizing enzymes) to produce the nascent peptidoglycan chain (Hiramatsu 2001). Again, clinical isolates of Vancomycin Intermediate *S. aureus* (VISA) and Vancomycin-Resistant *S. aureus* (VRSA) MRSA have been reported (Hiramatsu et al. 1997, Chang et al. 2003, Menichetti 2005). VRSA resistance is mediated by the acquisition of the VanA gene which originates from enterococci (Lowy 2003, Weigel et al. 2003). In the presence of the *vanA* gene in transposon Tn*1546*, enterococci can replace the DDR of peptidoglycan with D-Ala-D-

Lac (an alternative peptidoglycan to which vancomycin will not bind) to prevent their cell wall components from being bound to vancomycin (Cetinkaya et al. 2000, Tenover 2006) (see Figure 1.3 below).

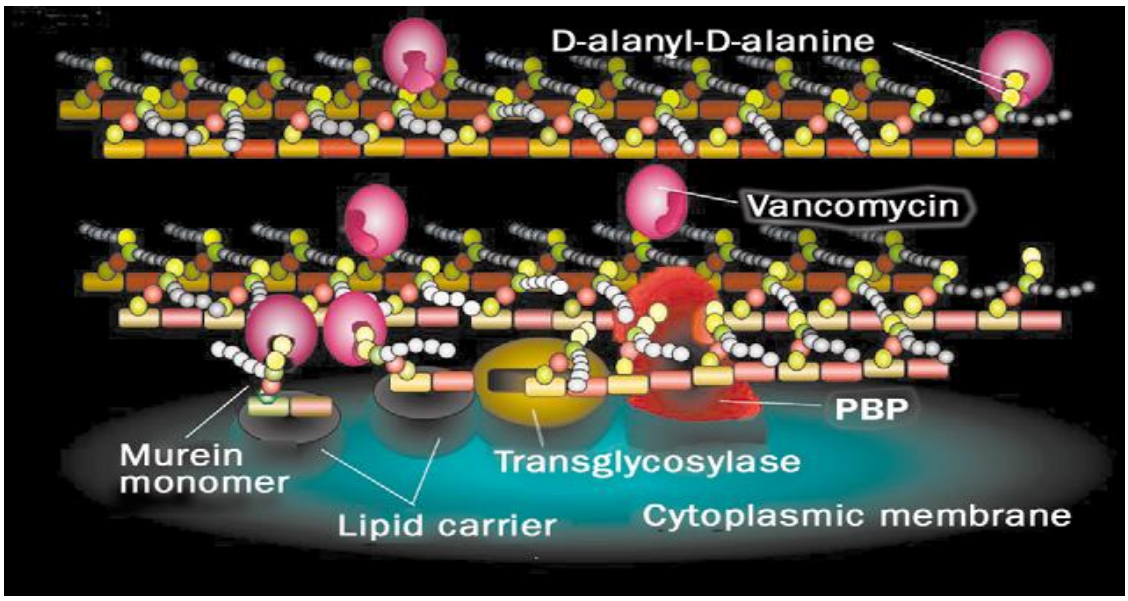


Figure 1.3 Action of vancomycin.
The antibiotic binds to D-alanyl- D-alanine residues of the murein monomer. The murein monomer bound by vancomycin does not serve as a substrate for glycosyltransferases. Source: (Hiramatsu, 2001).

The use of antistaphylococcal β-lactam antibiotics is still widely accepted as the standard of care to treat methicillin sensitive *S. aureus* (MSSA) whenever possible (Chang et al. 2003, Khatib et al. 2006, Kim et al. 2008, Schweizer et al. 2011). However, Vancomycin and daptomycin (a lipopeptide) are the only FDA-approved agents for the treatment of MRSA bacteremia in the United States and Teicoplanin is a potential alternative to Vancomycin (Holland et al. 2014). Today, several antibiotics are employed in the treatment of staphylococcal infections and combination therapy is often favoured over monotherapy. Therefore, high quality trials comparing treatment strategies, antibiotics and treatment durations are still needed to better inform the management of this common, serious infection and there is still an urgent need to search

for new novel anti-staphylococcal therapies. Teixobactin, a new antibiotic discovered more recently in uncultured bacteria, (Ling et al. 2015) is favoured as the next agent with potential for treatment of MRSA infections, pending FDA approval.

1.3.2 *Escherichia coli*

E. coli is a Gram-negative, non-sporulating, facultatively anaerobic rod, which either uses peritrichous flagella for mobility or is non-motile. They are about 2 µm long and 0.5 µm in diameter and grow in warm moist environments at 37°C (Hartl and Dykhuizen 1984). *E. coli* can be identified with a variety of biochemical reactions, the most useful being the indole test for the differentiation of *E. coli* from other members of the Enterobacteriaceae (Xia 2010). It can be recovered easily from clinical stool specimens on general or selective media (most often MacConkey or eosin methylene-blue agar) at 37°C under aerobic conditions (Balows 1991).

E. coli is a facultative anaerobe of the normal intestinal flora of humans and other mammals (Nataro and Kaper 1998, Kaper et al. 2004). *E. coli* has also been widely exploited as a cloning host in recombinant DNA technology. It is however more than just a laboratory model or harmless commensal. The organism typically colonizes the infant gastrointestinal tract within hours of life (Drasar and Hill 1974, Berg 1996) and remains harmlessly confined to the intestinal lumen. However, even "nonpathogenic" strains of *E. coli* can become versatile and deadly pathogens causing different infections in an immunosuppressed host or where the normal gastrointestinal barriers are breached (Nataro and Kaper 1998, Kaper et al. 2004, Croxen and Finlay 2010). The most common *E. coli* infections are diarrhoeal/enteric disease, meningitis/sepsis and urinary tract infection. It is also one of the leading causative agents of foodborne infections worldwide. Like most mucosal pathogens, *E. coli* infection follows colonization of a mucosal site, evasion of host defenses, multiplication, and host damage (Nataro and Kaper 1998).

E. coli implicated in human diseases are broadly classified into two major categories: the diarrhoeagenic *E. coli* and the extraintestinal pathogenic *E. coli* (Russo and Johnson 2000). Among the intestinal diarrhoeagenic *E. coli* pathogens, there are currently six well studied categories including Enteropathogenic *E. coli* (EPEC), Enterohaemorrhagic *E. coli* (EHEC)/Shiga toxin-producing *E. coli* (STEC), Enterotoxigenic *E. coli* (ETEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC) and Diffusely adherent *E. coli* (DAEC) (Nataro and Kaper 1998). Extraintestinal pathogenic *E. coli* (ExPEC) includes two major pathotypes: uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC) which colonize a variety of anatomical locations and cause various infections outside the gastrointestinal tract, among which urinary tract infections are the most common (Russo and Johnson 2000, Karch et al. 2005) (Figures 1.4 and 1.5 below illustrate the pathogenic schema of diarrhoeagenic and extraintestinal pathogenic *E. coli* respectively).

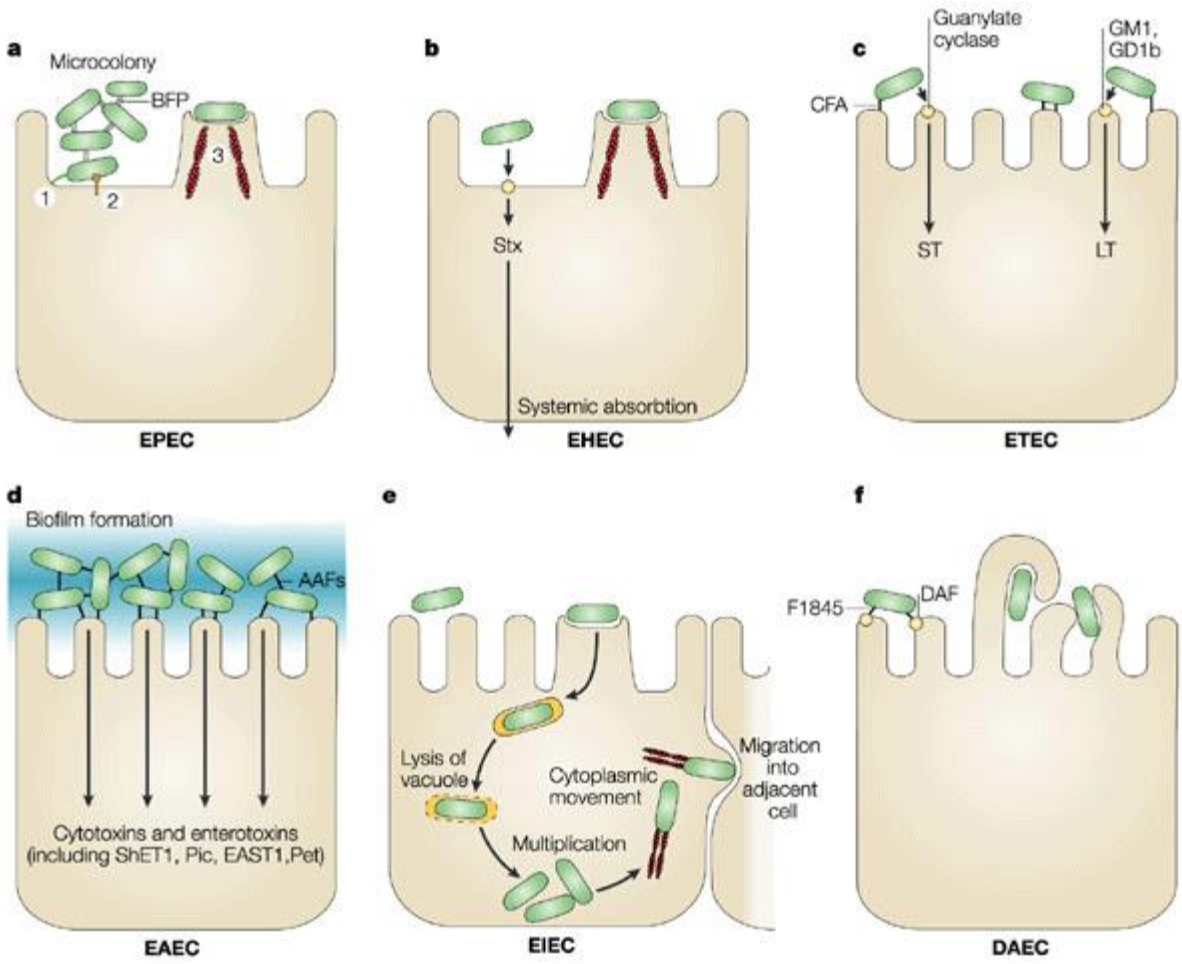


Figure 1.4 Pathogenic schema of diarrhoeagenic *E. coli.* Source: (Kaper et al. 2004). AAF, aggregative adherence fimbriae; BFP, bundle-forming pilus; CFA, colonization factor antigen; DAF, decay-accelerating factor; EAST1, enteroaggregative *E. coli* ST1; LT, heat-labile enterotoxin; ShET1, Shigellaenterotoxin 1; ST, heat-stable enterotoxin.

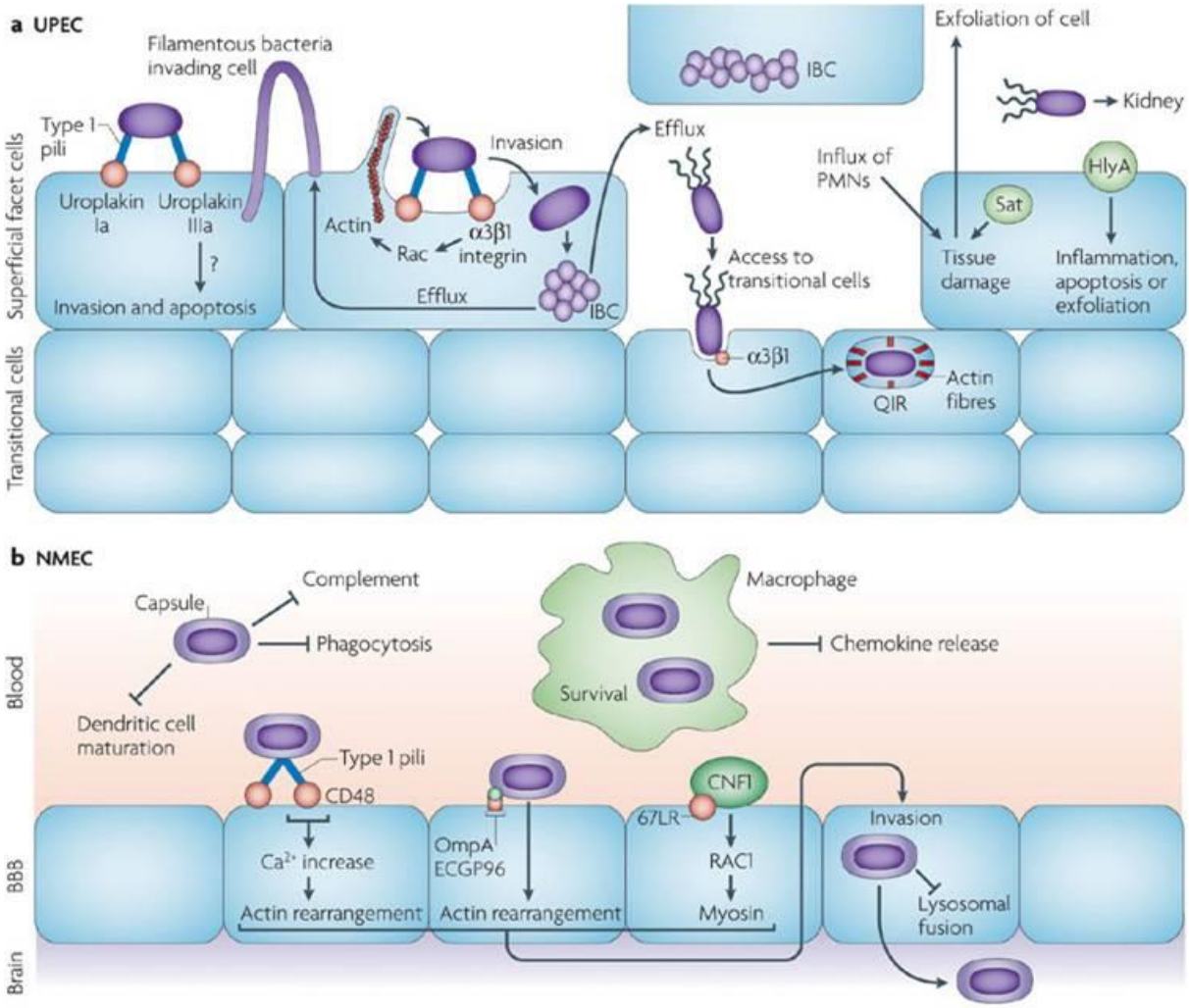


Figure 1.5 Pathogenic schema of extraintestinal *E. coli.* Source: (Croxen and Finlay 2010). **a** Uropathogenic *E. coli* (UPEC) attaches to the uroepithelium through type 1 pili, which bind the receptors uroplakin Ia and IIIa; this binding stimulates unknown signalling pathways (indicated by the question mark) that mediate invasion and apoptosis. Binding of type 1 pili to α3β1 integrins also mediates internalization of the bacteria into superficial facet cells to form intracellular bacterial communities (IBCs) or pods. Sublytic concentrations of the pore-forming haemolysin A (HlyA) toxin can inhibit the activation of Akt proteins and leads to host cell apoptosis and exfoliation. Exfoliation of the uroepithelium exposes the underlying transition cells for further UPEC invasion, and the bacteria can reside in these cells as quiescent intracellular reservoirs (QIRs) that may be involved in recurrent infections. **b** Neonatal meningitis *E. coli* (NMEC) is protected from the host immune response by its K1 capsule and outer-membrane protein A (OmpA). Invasion into macrophages may provide a replicative niche for high bacteraemia, allowing the generation of sufficient bacteria to cross the blood–brain barrier (BBB) into the central nervous system. Attachment of NMEC is mediated by type 1 pili binding to CD48 and OmpA binding to ECGP96. Invasion involves cytotoxic necrotizing factor 1 (CNF1) binding to 67 kDa laminin receptor (67LR; also known as RPSA, as well as type1 pili and OmpA binding their receptors. PMN, polymorphonuclear leukocyte; Sat, secreted autotransporter toxin.

E. coli strains cause diverse intestinal and extraintestinal diseases by means of virulence factors that affect a wide range of cellular processes. These virulence factors are normally encoded on genetic elements that can be exchanged between different strains or could be mobile elements which later become fixed into the genome. The versatility of the *E. coli* genome is conferred mainly by two genetic configurations: virulence-related conserved plasmid families and chromosomal pathogenicity islands, each encoding multiple virulence factors (Wood et al. 1986, Nataro et al. 1987, Hales et al. 1992). Although plasmids and pathogenicity islands carry clusters of virulence traits, individual traits may be transposon encoded e.g. heat-stable enterotoxin (ST) or phage encoded like Shiga toxin (So and McCarthy 1980, O'brien et al. 1992). There are also hundreds of serotypes of *E. coli* which are classified on the bases of various surface antigens referred to as Somatic (O), Capsular (K), Flagellar (H) and Fimbrial (F) (Edwards and Ewing 1972, Small and Falkow 1986, Lior 1994) (Table 1.2).

E. coli diarrhoea infections caused by various diarrhoeagenic *E. coli* normally do not normally require antibiotic treatment (Xia 2010). Potentially fatal infections such as infant meningitis and severe infections are generally treated with antibiotics. Commonly prescribed antibiotics include fluoroquinolones and cephalosporins and nitrofurantoin (Fadda et al. 2005). Although antibiotics have been very useful in treating *E. coli* infections in the past, the increase in antibiotic resistance in *E. coli* make treatment of *E. coli* infections complicated.

Table 1.2 Virulence factors in *E. coli* infection pathotypes. (Adapted from (Nataro and Kaper 1998, Clarke et al. 2003, Johnson and Russo 2005, Clermont et al. 2007, Wiles et al. 2008, Johnson and Nolan 2009, Abraham 2011, Santos et al. 2013)).

Pathotype	Associated H antigens	Virulence genes	Serotypes	Plasmids
ETEC	H9, H27, H11, NM, H42, NM, H7, H11, H12, H7, H28, H10, H20,NM	*Sta, Stb, Eastl, F4 (K88) F18, F41* (in neonatal diarrhoea) including *F5 (K99), F6 (987P)* (in post-weaning diarrhoea)	O6, O8, O9, O11, O15, O20, O25, O27, O45, O64, O78, O101, O128, O138,O141, O147, O148, O149, O157, O157, O173	FIB, FIIA, I1, FIC
ExPEC	H7 (generally less defined)	*Fimh, papG I to III, flu, focG, sfaS, afa/draBC, hlyA, cnfl, fyuA, iutA, iroN, kpsMT II, kpsMT III, kpsMT K1, kpsMT, traT K5, iss, irp2,* (in urinary tract infections)	O1, O2, O4,O6,O15, O18, O25,O75	FIB, FIIA, I1, FIC,Y B/O
EPEC	H34, NM, H2, H12, NM, H6, NM, H21, H27, NM, H6, NM, H2, H12, H6	*BfpA to L, bfpU, bfpP, bfpT, bftV, bfpW, escC, D, F, J, N, escR to V, sepQ, sepZ, Eae, tir, espA, B, D, F, G, map, ler, cesD, T, F.*	O55, O86, O111, O119, O125ac, O126, O127, O128, O142	com9 (pIP71A), IncA/C (pSN254), IncB/O (pTP113), IncD, IncFIA (Plasmid F), IncFIB (pO157), IncFIC (Plasmid F), IncFIIA (pR100), IncFIV (pSU316), IncFV (pED208), IncFVI (pSU212), IncFVII (pSU221). IncHI1 (pR27), IncHI2 (pR478), IncHI3 (Mip233), IncHII (pHH1508a), IncI1 (pR64), IncI2 (R721), IncJ (ICE R391), IncK (pR387), IncL/M (pCTX-M3), IncN (R46), IncP-α (RP4), IncP-β (pB4), IncP-γ (pQKH54), IncP-δ (pEST4011), IncP6 (IncG) (Rms149), IncP7 (pCAR1), IncP9 (pWWO), IncQ1 (RSF1010), IncQ2 (pTC-F14), IncR (pK245), IncT (pRts1), IncU (pFBAOT6), IncV, IncW (pR7K), IncX1 (pOLA52), IncX2 (pR6K), IncY (P1)

Antibiotics which were once effective in the treatment of *E. coli* infections including fluoroquinolones (e.g. ciprofloxacin- widely used oral antibacterial drug in the community) and cephalosporins (widely used for intravenous treatment of severe infections in hospitals) have become increasingly less effective (Besser et al. 1999). Multidrug resistance (MDR) to three or more drugs including streptomycin, cephalothin, tetracycline, ampicillin and trimethoprim has been reported (Taye et al. 2013, Ansari et al. 2015). AmpC-mediated β-lactam resistance in *E. coli* is an emerging problem (Odeh et al. 2002, Philippon et al. 2002, Wong-Beringer et al. 2002). High level AmpC production is typically associated with *in vitro* resistance to all β-lactam antibiotics except for carbapenems and cefepime. In addition, treatment failures with broad-spectrum cephalosporins have been documented (Odeh et al. 2002, Wong-Beringer et al. 2002). According to the World Health Organisation (WHO) Antimicrobial Resistance Global Report on Surveillance 2014, resistance in *E. coli* to fluoroquinolones, broad-spectrum penicillins (e.g. ampicillin or amoxicillin) and third-generation cephalosporins is widespread (WHO. 2014). Resistance to third-generation cephalosporins is mainly conferred by extended spectrum beta-lactamases (ESBLs) which destroy many beta-lactam antibacterial drugs. Carbapenems usually remain the only available treatment option for severe infections, as *E. coli* strains that have ESBLs are generally also resistant to several other antibacterial drugs. Moreover, carbapenem resistance in *E. coli,* mediated by metallo-betalactamases, which confer resistance to virtually all available beta-lactam antibacterial drugs, is a recently emerging medical threat (Menashe et al. 2001, Rawat and Nair 2010, Picozzi et al. 2013). Considering the limited therapeutic options for infections caused by some *E. coli* strains (Russo and Johnson 2006), research on developing effective vaccines and identifying risk factors in *E. coli* infections with an aim to prevent infections are ongoing.

1.3.5 *Enterococcus faecalis*

Enterococcus faecalis is a non-motile, Gram-positive, facultatively anaerobic lactic acid bacterium of about 0.6-2.0 µm in size, that appear singly, in pairs and in short chains under the microscope (Leavis et al. 2006, Ch. Schroder et al. 2015). It tolerates a wide variety of growth conditions, including temperatures of 10°C to 45°C, hypotonic, hypertonic, acidic, or alkaline environments. Sodium azide and concentrated bile salts, which inhibit or kill most microorganisms, are tolerated by enterococci and used as selective agents in agar-based media (Huycke et al. 1998). Although all enterococci are usually considered strict fermenters because they lack a Kreb's cycle and respiratory chain (Willett 1992), *Ent. faecalis* is an exception since, exogenous hemin can be used to produce cytochromes which provide a growth advantage to *Ent. faecalis* during aerobic growth (Pritchard and Wimpenny 1978, Brugna et al. 2010). Enterococci are usually catalase negative but some strains occasionally produce pseudocatalase.

Ent. faecalis is normally a gut commensal and is found in many animals (from cockroaches to humans) and in the environment (Huycke et al. 1998). It is also a frequent cause of many serious human infections, including urinary tract infections, endocarditis, bacteremia, and wound infections alongside *Ent. faecium* (Rice et al. 1995, Huycke et al. 1998, Kau et al. 2005). *Ent. faecalis* causes a variety of healthcare associated infections (HCAIs) of which urinary tract infections are the most common, responsible for approximately 110,000 cases yearly (Huycke et al. 1998, Kau et al. 2005). Enterococci are the second most common organisms recovered from catheter-associated infections of the bloodstream and urinary tract, and from skin and soft-tissue infections in hospitals in the United States (Arias and Murray 2008, Hidron et al. 2008).

Infections with *Ent. faecalis* can be especially troublesome to treat because of their frequent resistance to multiple antibiotics, including vancomycin, a drug of last resort for many Gram positive infections (Huycke et al. 1998). Enterococci are also relatively

impermeable to aminoglycosides, and the serum concentrations of aminoglycosides required for bactericidal activity are much higher than can be achieved safely in humans. However, the simultaneous use of a cell wall-active agent raises the permeability of the cell so that an intracellular bactericidal aminoglycoside concentration can be achieved without excessive toxicity (Baddour et al. 2005). Linezolid inhibits the growth of *Ent. faecalis* but quinupristin-dalfopristin (Synercid) does not because *Ent. faecalis* are intrinsically resistant to quinupristin-dalfopristin. *Ent. faecalis* are also often resistant to kanamycin and amikacin although many isolates still exhibit some susceptibility to ampicillin and penicillin. Surgery and cardiac valve replacement may be the only chance of cure in some patients with endocarditis resulting from enterococci for which there is no synergistic bactericidal combination (Baddour et al. 2005).

Enterococci are intrinsically resistant to many antibiotics (Dunny et al. 1995, Huycke et al. 1998). Unlike acquired resistance and virulence traits, which are usually transposon or plasmid encoded, intrinsic resistance is based in chromosomal genes, which typically are nontransferrable. The first reports of strains resistant to penicillin began to appear in the 1980s (Bush et al. 1989, Sapico et al. 1989). Enterococci often acquire antibiotic resistance through exchange of resistance-encoding genes carried on conjugative transposons (Chavers et al. 2003). Detailed reviews on enterococcal resistance mechanisms are ample in literature (Arias and Murray 2012, Hollenbeck and Rice 2012, Kristich et al. 2014) (Figure 1.6). Vancomycin-resistant enterococci, VRE (Uttley et al. 1989), in particular *Ent. faecium*, are often multidrug-resistant, however, vancomycin-resistant *Ent. faecalis* are usually penicillin susceptible. Susceptibililty, intermediate level resistance and complete resistance to vancomycin are considered as MICs $\leq$ 4 µg/ml, 8-16 µg/ml and >16 µg/ml respectively. Five phenotypes of vancomycin resistance VanA B, C, D and E in enterococci have been described (Chavers et al.

2003). VanA and VanB phenotypes are more clinically important and result from the acquisition of new genetic determinants of resistance carried on transposon Tn *1546* (Cetinkaya et al. 2000). The genes encoding VanA and VanB are found primarily in *Ent. faecium* and some strains of *Ent. faecalis.* Infective Endocarditis (IE) cases are often caused by the VanA, B, or C phenotypes (Cetinkaya et al. 2000, Chavers et al. 2003). The VanA phenotypes display a high-level of vancomycin resistance (MIC >64 µg/ml) and teicoplanin resistance. The VanB phenotypes have intermediate to high-level vancomycin resistance (MIC 4 to 1024 µg/ml) and susceptibility to teicoplanin, while and VanC phenotypes possess low to intermediate level Vancomycin resistance (MIC 2 to 32 µg/ml) (Chavers et al. 2003).

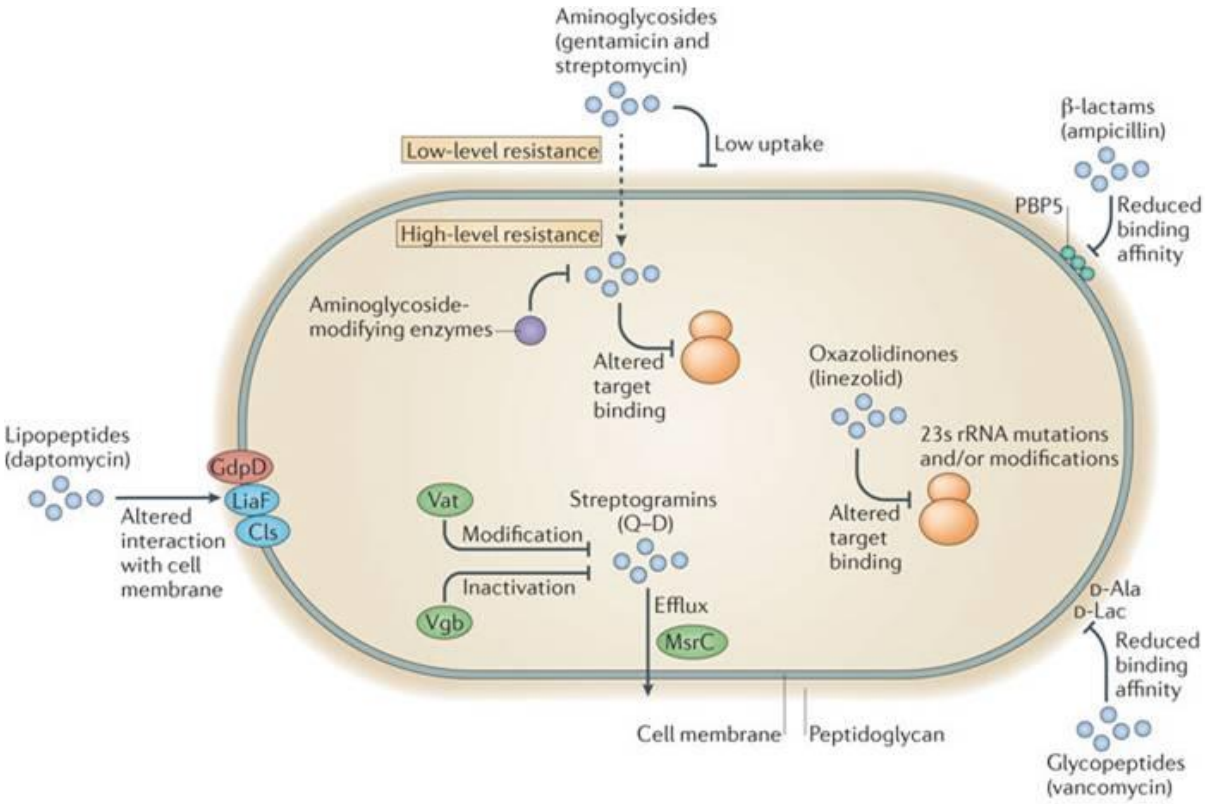


Figure 1.6 Mechanisms of enterococcal antibiotic resistance. Source: (Arias and Murray 2012). Resistance of *Ent. faecalis* to the lipopetide daptomycin has been shown to involve altered interactions with the cell membrane and requires the membrane protein LiaF and enzymes involved in phospholipid metabolism, such as a member of the glycerophosphoryl diester phosphodiesterase family (GdpD) and cardiolipin synthase (Cls).

VRE are significant multidrug-resistant opportunistic pathogens in the hospital environment and the potential spread of enterococcal vancomycin resistance determinants to other species remains a concern (Chang et al. 2003). Wiser use of antimicrobial drugs, possibly guided by novel techniques for rapid microbiological diagnosis, and the nascent trend toward the development of narrower-spectrum antimicrobials may diminish some of the selective pressures favouring VRE. Continued development of new drugs by the pharmaceutical industry, aided by genomics, high-throughput screening, rational drug design, and novel therapies offer the best prospect of effective bactericidal monotherapy for enterococci and long-term solutions to the problem of VRE (Eliopoulos and Gold 2001).

1.4 Discovery of novel antimicrobials

Antibiotic resistant strains of pathogens, often with multiple antibiotic resistance continue to emerge (House of Lords Select Committee for Science and Technology (HLSCST) 1998) and there is decreased discovery of novel antibiotics (Mollering 1995). This means that the rate of loss of efficacy of old antibiotics is outstripping their replacement with new ones for many species of pathogenic bacteria (Hancock 2007). The traditional answer to the problem of antimicrobial resistance has been to introduce new antimicrobial agents and alternative treatments that kill the resistant mutants. Unfortunately, after more than 50 years of success, the pharmaceutical industry is now producing too few antibiotics, particularly against Gram-negative organisms to replace antibiotics that are no longer effective for many types of infections.

Although analogues of existing antibiotic families kill resistant bacteria and prolong the life of each family for decades, this well of discovery will eventually dry out (Coates and Hu 2007). Therefore, the discovery of novel families of antimicrobials at regular intervals is important if modern medicine is to continue in its present form. This is

because growth on media has been an essential step to the development of antibiotics and to date, antibiotics such as streptomycin have been derived from bacteria that grow on artificial solid or liquid media. However, as most species of bacteria will not grow on artificial media (Diaz-Torres et al. 2006), antimicrobials are yet to be isolated from non-culturable bacteria. Nevertheless, it is now possible to clone large fragments of non-culturable bacterial genomes and to express them using recombinant DNA technology (Garcia Martin et al. 2006, Jones and Marchesi 2007, Lee et al. 2007) (Figure 1.7 below shows one method by which this can be achieved). Since the screening of whole bacteria against novel, natural and chemical compound libraries may produce new antibiotics, non-culturable bacteria such as those from the rumen may be an alternative source for novel compounds (Coates and Hu 2007).

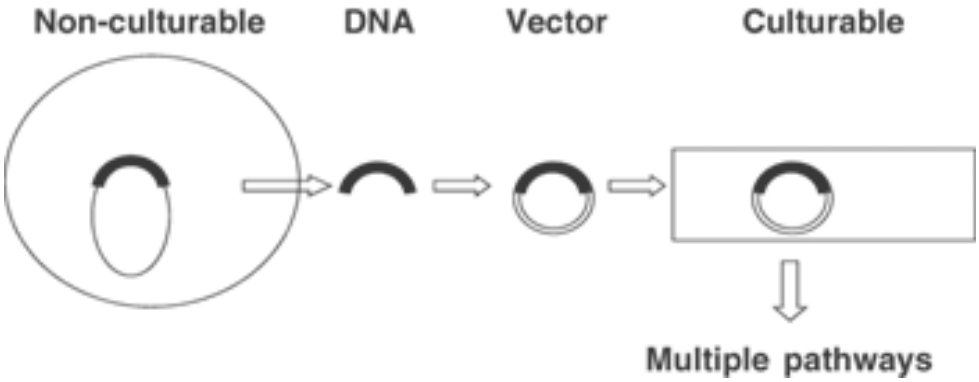


Figure 1.7 A method for screening non-culturable bacteria for novel compounds. The unfilled circle on the left of the diagram depicts the non-culturable bacterium. Within the circle is the smaller ring, which shows the bacterial genome with the thick black segment representing the coding region for synthesis of a novel antibiotic. The DNA of the coding region is extracted, inserted into a vector and this is transfected into a culturable bacterium or other host that expresses the antibiotic. Antibiotic biosynthesis often involves multiple pathways that are found on relatively large genomic segments of DNA, and cloning these pathways is often a complex task. (Adapted from (Coates and Hu 2007)).

1.5 Introduction to ruminants

Herbivores play a central role in the survival and well-being of mankind. In addition to food, herbivorous animals are sources of clothing and motive power for transport and other activities of humans (Hobson and Stewart 1997, Russell and Rychlik 2001). The group of herbivores mostly used by humans are the ruminants (Russell and Rychlik 2001). Ruminants of agricultural importance are mainly cattle, sheep, deer and goats, and of these, the most attention has been paid to the first two species. While humans and other animals have digestive systems with enzymes which digest animal and plant protein and lipids, plant sugars and some polysaccharides such as starches, they have poor mechanisms for digesting the much more abundant structural polysaccharides of plants: cellulose, hemicelluloses and pectins, all of which can be digested by ruminants (Balch 1959, Hungate 1966). Ruminant species occupy an important niche in agriculture because of their unique ability to digest roughages (plant cellulose, hemicellulose and pectins) (Bowen 2006, Brulc et al. 2009). The ruminant has three compartments in its digestive tract before the true gastric stomach (abomasum). These are the reticulum, rumen and omasum (Balch 1959, Hobson and Stewart 1997) (Figure 1.8).

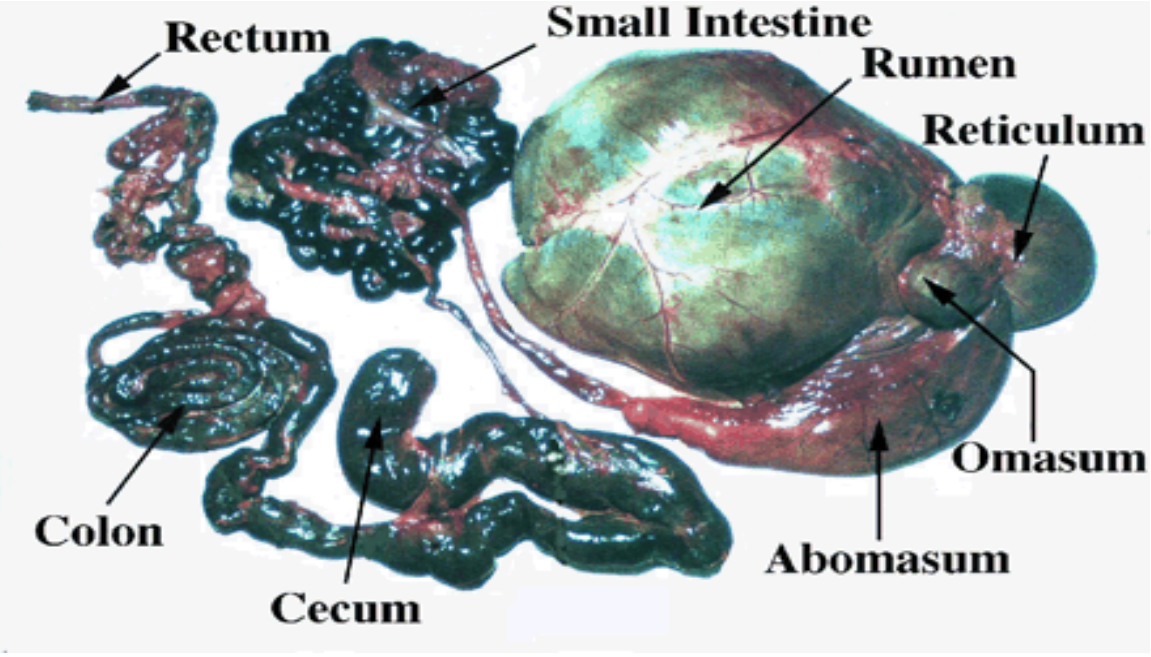


Figure 1.8 The digestive tract of a healthy adult ruminant showing the various compartments (reticulum, rumen, omasum, abomasum, small intestine, cecum, colon, and rectum**)**. Source: (Russell 2000).

1.5.1 The reticulum

The reticulum is a flask-shaped compartment with a "honeycomb" appearance (Bowen 2003, Visser 2005) (Figure 1.9). It sits underneath and towards the front of the rumen lying against the diaphragm. Ingesta flow freely between the reticulum and rumen, as they are only separated by a small muscular fold of tissue and are sometimes considered as one organ, referred to as the reticulorumen (Parish 2011). The main function of the reticulum is to collect smaller digesta particles and move them into the omasum while the larger particles remain in the rumen for further digestion (Parish 2011). The reticulum also causes the regurgitation of ingesta during rumination and acts as a collection compartment for foreign objects (Wester 1929, Visser 2005).

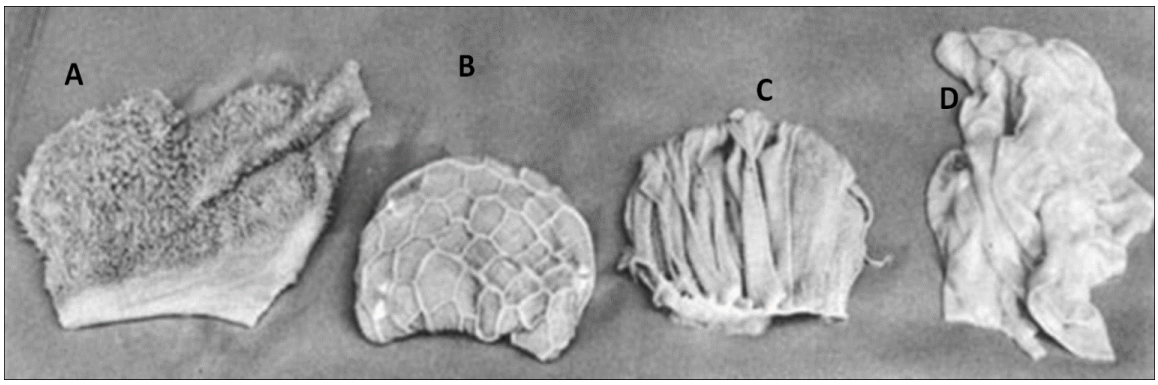


Figure 1.9 Inside structures of the ruminant stomach. Showing (A), papillae lining of the rumen (B), honeycomb appearance of the reticulum (C), many folds- 'manypiles' of the omasum and (D), the abomasum (Source: (Correa 2007).

1.5.2 The rumen

The rumen resembles a large fermentation vat of 100 or more litres in volume in adult cattle and 10 litres in sheep (Hobson and Stewart 1997, Visser 2005, Bowen 2009). It hosts a large population of microorganisms, especially bacteria, ciliated protozoa and anaerobic fungi, which digest cellulose from plant cells (Hobson and Stewart 1997, Kamra 2005). The rumen environment is anaerobic and it is lined with papillae for nutrient absorption (Figure 1.9). The tissue of the rumen and the omasum absorb volatile fatty acids (VFAs), which are the by-products of microbial fermentation. Gases such as carbon dioxide, methane and hydrogen sulphide are also produced in the rumen. For optimum ruminal fermentation and fiber digestion, ruminal pH should lie between 6.0 and 6.4, although, even in healthy cows, ruminal pH will fluctuate below this level for short periods during the day (Mutsvangwa and Wright 2012). The environment in the reticulorumen is however, weakly acidic and is populated by microbes that are adapted to a pH roughly between 5.5 and 6.5 (Dijkstra et al. 2005, Mutsvangwa and Wright 2012). Also, in normal conditions, the ruminal milieu is anaerobic (as any ingested oxygen is quickly scavenged) with a markedly negative redox potential, reflecting the absence of oxygen and a strong reducing power (Marden et al. 2005).

1.5.3 The omasum

The Omasum or "manyplies", so called because it contains numerous tissue leaves

(laminae) that help grind digesta (Parish 2011) (Figure 1.9), is spherical in shape and is situated to the right side of the abdomen (Balch 1959, Nickel et al. 1987). Water and short-chain fatty acids absorption occurs in the omasum and the folds assist in the removal of fluid from the digesta on their way to the abomasum (Dirksen 1990). The omasum also serves to filter large particles which return to the rumen for further digestion (Hungate 1988).

1.5.4 The abomasum

The abomasum is the "true stomach" of a ruminant and corresponds to the gastric stomach of non-ruminants (Figure 1.9). It secretes hydrochloric acid and other digestive enzymes, which aid in digestion. The low pH in the abomasum (ranges from 2.0 to 4.0) facilitates initial protein breakdown and kills the microorganisms, which have spilled over from the rumen (Visser 2005, Parish 2011).

1.6 The rumen microbial ecosystem

In young ruminants, small pieces of vegetation together with some milk enter the developing rumen through contact from the mother and surroundings and become substrates for microbes (Hobson and Stewart 1997). The rumen is subject to continuous inoculation with microorganisms of many different kinds, from many sources (Hungate 1966). The rumen inoculum can be provided by airborne transfer from nearby adult animals or implements and clothing of farm workers moving between areas of young and old stock, from saliva and faeces of mother and other animals in the group, and contaminated vegetation (Hungate 1988). It is possible to isolate many species of microorganism from the rumen. However, many of these will be in very low numbers and merely passing through the rumen and dying off, as they may not be adapted for life in the rumen. On the other hand, others will be 'true' rumen organisms and will replace dying components of the rumen flora and contribute to the 'dynamic steady state' of the

rumen population (Stewart et al. 1992, Odenyo et al. 1994, Hobson and Stewart 1997, Kamra 2005).

The efficiency of ruminants to utilize such a wide variety of feeds is due to the highly diversified rumen microbiome consisting of bacteria (~ $10^{10\text{-}11}$ ml^{-1}), ciliate protozoa (~ 10^{6} ml^{-1}), anaerobic fungi (~10^{5} ml^{-1}), and bacteriophages (10^{8} ml^{-1}) (Hespell et al. 1987, Klieve and Bauchop 1988, Hobson and Stewart 1997, Sylvester et al. 2004, Wright and Klieve 2011). Nonetheless, this is not a true representation of the number of rumen microbes as many of them are non-culturable. In one study (Edwards et al. 2004), only 11% of the bacterial diversity was represented by cultured isolates, and of the 11%, not all of the representative cultured isolates were of ruminal origin. Microbial population numbers and composition are affected by a number of factors of which diet is probably the most important. Smaller numbers of facultative anaerobic bacteria, flagellate protozoa and mycoplasmas are also believed to be present but their contribution to the overall fermentation is considered minimal (Dehority and Orpin 1997).

The net result of the reactions carried out by the complex microbiome of the rumen is the bioconversion of feed into such form as is utilizable by the animal as a source of energy e.g. microbial protein, short chain volatile fatty acids including acetic, propionic and butyric acids and gases such as methane and carbon dioxide (Kamra 2005, Brulc et al. 2009).

1.6.1 Rumen bacteria

Based on standard physiological characteristics, the microbial ecosystem of the rumen typically harbours representatives of several divergent taxonomic groupings of bacteria, which are active in the degradation of feed components (Stewart et al. 1992, Krause and Russell 1996, Kamra 2005). Strictly anaerobic techniques and habitat simulating media

have been used to isolate a variety of bacteria from the rumen (Hungate 1944, Hungate 1950, Hungate 1969, Dehority et al. 1989). More recently, ruminal bacteria have been re-evaluated with newer more objective methods of classification (Krause and Russell 1996, Deng et al. 2008). It is not yet possible to clearly answer the question of how many rumen bacterial species there are (Krause and Russell 1996), however, knowledge of evolutionary conservation of 16S/18S ribosomal RNA (rRNA) and their encoding genes (rDNA) (Pace et al. 1986, Wheelis et al. 1992, Raskin et al. 1997) has facilitated the use of modern molecular techniques to enumerate microbes. Molecular characterisation and classification scheme, which predict natural evolutionary relationships without cultivating the organisms is now possible (Mackie et al. 2000).

The wide range of aerobic, facultative and anaerobic organisms found especially in young ruminants have been reviewed (Bryant et al. 1958, Hungate 1966, Jayne-Williams 1979, Stewart et al. 1988). Anaerobic species found in relatively large numbers include *Bifidobacterium* sp. (Scardovi 1981), *Peptostreptococcus* sp. (Bryant et al. 1958, Jayne-Williams 1979), *Propionibacterium acnes* (Gutierrez 1953) and many clostridia and *Bacteriodes*. *Alysiella filiformis* is a strict aerobe found on rumen epithelia of young lambs (Mueller et al. 1984). It is more commonly found in the buccal cavity, but can occasionally establish on the rumen epithelium as a transient colonizer (Mueller et al. 1984, Mueller et al. 1984). Other aerobic organisms isolated from the rumen are *P. aeruginosa, Micrococcus varians, Alkaligenes faecalis, Flavobacterium* spp. and several spore-forming *Bacillus* species. Staphylococci and streptococci are the genera of facultative anaerobes most frequently found in the rumen. Others include *Ent. faecium.* Coliforms are also present in low numbers.

Rumen bacteria vary greatly in their substrate specificity. Some, such as, *Ruminobacter amylophilus* and *Fibrobacter succinogenes* are specialists, which utilize mainly starch

or its degradation products, and cellulose or its breakdown products respectively (Russell et al. 2009, Wanapat and Cherdthong 2009). Others like strains of *Butyrivibrio fibrisolvens* are generalists, which hydrolyse a range of substrates including starch, cellulose, xylan and pectin. Others like *Selenomonas ruminantium* can only utilize hydrolysis products, being unable to hydrolyse many polymers. *Oxalobacter formigenes* obtains energy from the reduction of oxalate. Tables 1.3 to 1.5 show the main characteristics of predominant rumen bacteria.

However, the functionality of most rumen bacteria is not known because most of them cannot be grown in the laboratory. It is estimated that only about 15- 20% of rumen microbes have been identified and characterised to date using conventional culturing and microscopy (Sirohi et al. 2012), and although large numbers of bacterial operation taxonomic units (OTUs) have been identified by ribosomal RNA gene (*rrn)* sequences, more species-level OTUs remain to be identified (Kim et al. 2011). It has also been shown that the diversity of estimates for all bacterial groups in the rumen are much greater than previously suggested (Edwards et al. 2004, Yu et al. 2006, Kim et al. 2011). Moreover, in another study (Kim et al. 2011), it was concluded that to achieve 99.9% coverage at species level, at least 78218 bacterial sequences would be needed in addition to the 13478 *rrn* sequences found in their study.

Table 1.3 Bacterial diversity of the rumen microbial ecosystem of domestic and wild animals (Adapted from (Kamra 2005)).

Substrate	Bacteria
Bacteria active in carbohydrate utilization:	
Cellulose	*F. succinogenes, Ruminococcus flavefaciens, R. albus, Clostridium cellobioparum, C. longisporum, C. lochheadii, Eubacterium cellulosolvens*
Hemicellulose	*Buty. fibrisolvens, Prevotella ruminicola, Eub. xylanophilum,, Eub. uniformis*
Starch	*Streptococcus bovis, Rum. Amylophilus, Prev. ruminicola*
Sugars/dextrins	*Succinivibrio dextrinosolvens Succ. amylolytica Sele. ruminantium , Lactobacillus acidophilus, L. casei, L. fermentum,, L. plantarum, L. brevis,, L. helveticus Bifidobacterium globosum, B. longum,, B. thermophilum B. ruminale, B. ruminantium*
Pectin	*Treponema saccharophilum, Lachnospira multiparus*
Bacteria active in nitrogen utilization:	*Prev. ruminicola, Rum. amylophilus, C. bifermentans*
Protein degraders	*Megasphaera elsdenii*
Other bacteria:	
Acid utilizers	*M. elsdenii, Wollinella succinogenes, Veillonella gazogenes, Oxal. formigenes, Desulphovibrio desulphuricans, Desulphatomaculum ruminis, Succiniclasticum ruminis*
Lipolytic bacteria	*Anaerovibrio lipolytica*
Acetogenic bacteria	*Eub. limosum, Acetitomaculum ruminis*
Tannin degraders	*Strep. Caprinus, Eub. oxidoreducens*
Mimosine degraders	*Synergistes jonesii*
Mycoplasma	*Anaeroplasma bactoclasticum, Anaep. abactoclasticum*

Table 1.4. Fermentation characteristics of some rumen cocci. Source: (Hobson and Stewart 1997). Abbreviations: A, acetate; B, n-butyrate; F, formate; iB, isobutyrate; iV, isolvalerate; L, lactate; P, propionate; S, succinate; V, n-valerate +, positive reaction; -, negative reaction; d, reaction varies between strains; blank, not tested; NR, not recorded

	Ruminococcus albus	*R. flavefaciens*	*R. bromii*	*Streptococcus bovis*	*Megasphaera elsdenii*
Cell wall Gram type (by EM)	+	+	+	+	–
Acid from:					
Starch	–	–	+	+	–
Cellulose	+	+	–	–	–
Xylan	+	+	–		
Pectin				d	–
Maltose	–	–	+	+	+
Cellobiose	+	+	–	+	–
Sucrose	d	d	–	+	d
D-Xylose	d	d	–	d	–
L-Arabinose	d	d	–	d	–
Glucose	d	d	d	+	+
Fructose	d		+	+	+
Galactose	–	–		+	–
Mannose	d	d	d	+	–
Lactose	d	d	–	+	–
Mannitol	d	–	–	d	+
Glycerol	–	–	–	d	d
Lactate	–	–	–	–	+
Aesculin hydrolysis	d	d		d	–
H_2S production	–	–	–		+
Nitrate reduction	–	–	–		–
Fermentation products:					
Major	A2	AS	A2	L	CB45
Minor/some strains	FL	FL	LFPB	FA2	iV V iB AS i4 i5 F P
Gas produced	H_2CO_2	H_2CO_2	H_2CO_2	CO_2	H_2CO_2
G + C (mol. %) in DNA	42–46	39–44	39–40	37–39	53–54
Type strain (ATCC)	27210	19208	27255[a]	33317	25940

[a] Not a rumen strain.

Table 1.5 Fermentation characteristics of some rumen Gram-positive rods. Source: (Hobson and Stewart 1997).

	Lachnospira multipara	*Lactobacillus ruminis*	*Lactobacillus vitulinus*	*Clostridium polysaccharolyticum*	*Clostridium longisporum*	*Eubacterium ruminantium*	*Eubacterium cellulosolvens*	*Butyrivibrio fibrisolvens*
Acid from:								
Starch	d			+	–	–	–	d
Cellulose	–			+	+	–	+	d
Xylan	–			+	–	d	d	+
Pectin	+			+	+	d	d	+
Maltose	d	+	+	+	–	d	+	d
Cellobiose	+	+	+	+	+	+	+	d
Sucrose	+	+	+	–	+	d	+	d
D-Xylose	d	–	–	+	–	d	–	d
L-Arabinose	–	–	–	+	–	d	–	+
Glucose	+	+	+	–	+	+	+	+
Fructose	+	+	+	d	+	+	+	+
Galactose	d	+	+	–	+	–	d	+
Mannose	d	+	+	–	+	–	–	+
Lactose	d	d	+	–	–	d	+	d
Mannitol	–	–	–	–	–	–	–	–
Glycerol	–	–	–	–		–	–	–
Lactate	–			–		–	–	–
Aesculin hydrolysis	+	+	+	+	+	d	d	d
H_2S production	–					–	–	–
Nitrate reduction	d			–	–	–	–	–
Fermentation products								
Major	FAL	L	L	FBA	FBA	BFL	L	FBA
Minor/some strains	S2			P2	2	AP	FASB	LS
Gas produced	H_2CO_2			H_2	H_2CO_2	CO_2	H_2	H_2CO_2
G + C (mol. %) in DNA	NR	44–47	34–37	42	33	NR	NR	36–41
Type strain (ATCC)	19207	27780	27783	33142		17233	B384	19171

1.6.2 Rumen protozoa

Rumen protozoa represent a substantial part of the rumen microbial population, contributing up to 50% of the total microbial biomass (Williams and Coleman 1992). Rumen protozoa were detected in domestic ruminants (Gruby and Delafond 1843) in the early nineteenth century. However, due to highly specific growth requirements and their complex morphology, our understanding of the role of protozoa in rumen fermentation is still limited. Ciliate protozoa in the rumen are classified on the basis of the micro- and macronucleus, the presence and morphology of exterior spines, lobes or internal skeletal plates and the shape and size of cells (Dogiel 1927, Ogimoto and Imai 1981, Williams and Coleman 1992). Based on such morphological characteristics a large number of genera and species have been described, but it is not clear to what extent these represent true species (Regensbogenova et al. 2004, Regensbogenova et al. 2004) and the validity of these observations has been questioned, because many of the 'species' described exhibit a substantial morphological plasticity (Dehority 1994).

The most prevalent protozoa of the rumen are the ciliated protozoa (Williams and Coleman 1997). Although flagellates like *Trichomonas* spp., *Monoceromonas* sp. and *Chilomastix* sp., have been reported, their status as "true" rumen residents is disputed because almost nothing is known of their metabolism (Jensen and Hammond 1964). The ciliate protozoa of the rumen have been classified into two groups, i.e. holotrich and entodiniomorphid protozoa (depending on their morphological characteristics), both of which belong to the subclass Trichostomatia (Hungate 1966). Ruminal holotrichs are represented by 15 different genera of ciliate protozoa that are classified within two separate taxonomic orders (Isotrichidae and Buetschliidae) (Williams and Coleman 1997) (Figure 1.10).

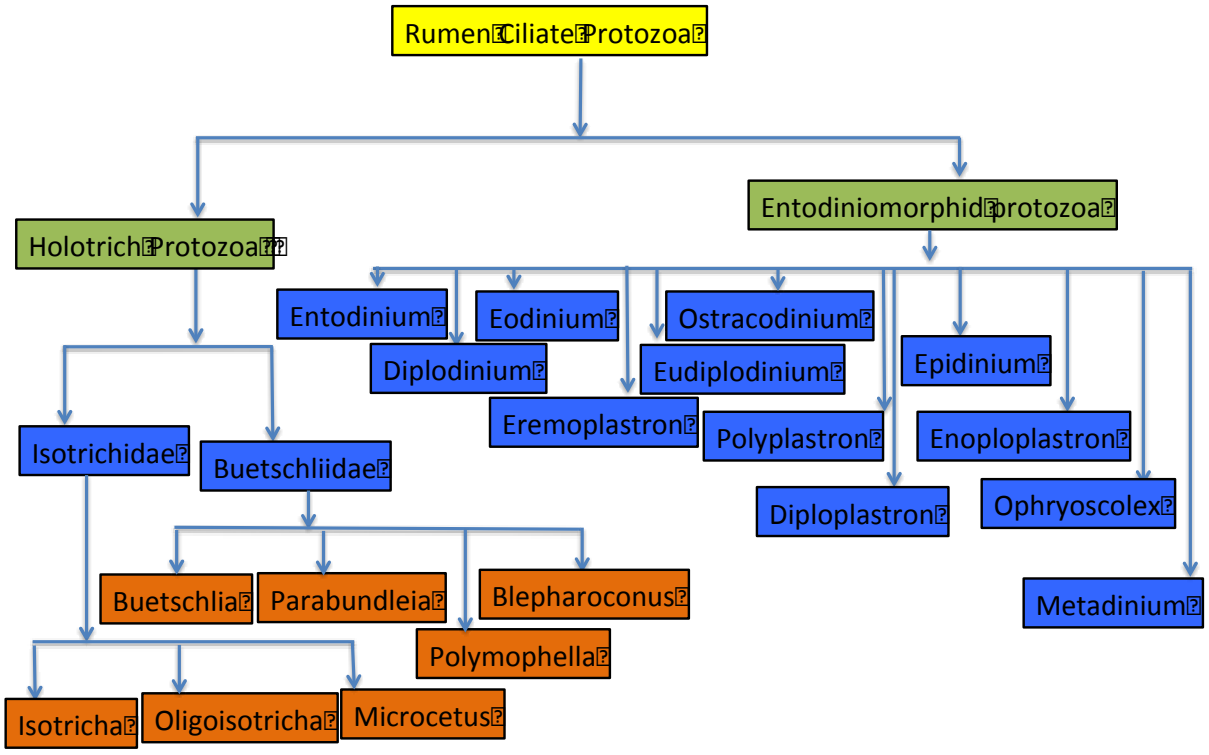


Figure 1.10 Phylogenic tree summarizing the major groups of rumen ciliate protozoa.

The enzymatic profile of holotrich protozoa indicates that they have amylase, invertase, pectin, esterase and polygalacturonase in sufficiently large quantities. These degrade pectin, which is an insoluble carbohydrate to enable utilisation of starch and soluble sugars as energy sources (Mould and Thomas 1958, Abou Akkada and Howard 1961, Bailey and Howard 1963, Williams 1979). Hemi-cellulases and cellulases have also been reported but in considerably lower levels compared to those present in entodiniomorphid protozoa (Mould and Thomas 1958, Abou Akkada and Howard 1961, Bailey and Howard 1963, Williams 1979, Williams and Coleman 1985). Entodiniomorphid protozoa also contain proteolytic enzymes and amino acid degraders (Abou Akkada and Howard 1961, Williams and Coleman 1992). A tryptophanase has been described in mixed rumen ciliates (Okuuchi et al. 1992).

1.6.3 Rumen anaerobic fungi

Yeasts and anaerobic fungi have long been recognized as typical inhabitants of the rumen. Flagellated anaerobic fungal zoospores were observed as early as 1910, but were mistakenly thought to be flagellate protozoa. These 'flagellates' were identified as fungi for the first time by (Orpin 1975) following new culture methods (Jensen and Hammond 1964), and were named as *Neocallimastix frontalis. N. frontalis* zoospores grew into mycelia which turned into a reproductive stage of rhizoids bearing zoosporangium. *N. frontalis* was confirmed to be a true fungus by the presence of chitin in its cell wall (Orpin 1977), and was the first strictly anaerobic fungus to ever be described. Several strains of anaerobic fungi have been reported in the rumen of different ruminants (Kamra 2005, Liggenstoffer et al. 2010). However, only six genera and 20 species of anaerobic fungi have been described, mainly on the basis of their morphological characteristics: *Anaeromyces, Caecomyces, Cyllamyces, Neocallimastix, Orpinomyces* and *Piromyces* (Griffith et al. 2009, Kittelmann et al. 2012). Two new genera *Oontomyces* and *Buwchfawromyces* have been identified in more recent studies (Callaghan et al. 2015, Dagar et al. 2015).

The anaerobic fungi found in the rumen play an active role in fiber degradation as evidenced by the presence of different fiber degrading enzymes including glycoside hydrolases, cellulases, xylanases, and esterases (Williams and Orpin 1987, Teunissen and Op den Camp 1993, Wubah et al. 1993, Paul et al. 2003). Fungi have also been shown to attach to the most lignified tissue of plant feed material (Akin and Rigsby 1987).

1.6.4 Rumen archaea

Rumen archaea are strictly anaerobic methanogens which make up between 0.3 and 3.3% of the rumen microbiome. The methanogens in the rumen are found free in the

rumen fluid, attached to particulate material, rumen epithelia and rumen protozoa (Janssen and Kirs 2008). There is also evidence that methanogens attach to anaerobic fungi (Jin et al. 2011). Methanogens that have been cultured from the rumen are assigned to seven species, i.e. *Methanobacterium formicicum, Me. bryantii, Methanobrevibacter ruminantium, Met. millerae, Met. olleyae, Methanomicrobium mobile, Methanoculleus olentangy*i (Paynter and Hungate 1968, Jarvis et al. 2000, Rea et al. 2007), although *Methanosarcina* spp. and *Met. smithii* have also been reported (Joblin et al. 1990, Joblin 2005).

The mixed microbial community ferments a large part of the incoming feed to volatile fatty acids (VFAs), NH_4^+, CO_2, and H_2. Some of these end products are metabolized by the methanogens, most notably CO_2, and H_2 (Janssen and Kirs 2008). Efficient H_2 removal leads to an increased rate of fermentation by eliminating the inhibitory effect of H_2 on the microbial fermentation (Hungate 1967, Wolin 1979, McAllister and Newbold 2008). Therefore, although methanogenic archaea make up only a small part of the rumen microbes, they play an important role in rumen function and nutrition (Kamra 2005, Janssen and Kirs 2008).

1.7 Rumen Microbial Interactions

A number of studies have been conducted on the interrelationships between rumen fungi, bacteria and protozoa (Dehority and Tirabasso 2000), and regardless of the complicated interrelationships between them, bacteria are believed to play a major role because of their numerical predominance and metabolic diversity (Windham and Akin 1984, Akin and Benner 1988, Cheng 1999). The role and importance of anaerobic fungi in the overall rumen fermentation remains a major question to be answered in spite of their unique ability to degrade the more resistant plant cell wall (Orpin 1994, Orpin 1997). A more recent analysis of muskoxen rumen metatranscriptome resulted in a

much higher percentage of cellulase enzyme discovery and an 8.7x higher rate of total carbohydrate active enzyme discovery per gigabase of sequence than previous rumen metagenomes (Qi et al. 2011). This study provided a snapshot of eukaryotic gene expression in the muskoxen rumen, and identified a number of candidate genes coding for potentially valuable lignocellulolytic enzymes (Qi et al. 2011).

The synergism and antagonism among the different groups of microbes that make up the cellulolytic biofilms in the rumen is so diverse and complicated that it is difficult to quantify the role played by particular groups, however, the net result of these reactions is the fermentation of cellulose and other plant material in the rumen (Stewart 1999, Lee et al. 2000, Kamra 2005). A consequence of this high level of diversity and population density of rumen microbes is increased opportunity for interaction between cells and presumably, intense competition between many organisms with similar phenotypes but different genotypes (Teather 1999). The fact that the rumen is a contained environment, with significant levels of mixing, means that there is opportunity for both direct and indirect interactions between all parts of the microbial population, and according to (Wolin et al. 1997), these metabolic interactions are essential for sustaining the microbial community and its collective activities. In general, interactions can be either positive or negative within and between microbial types. These associations have been described in three terms: mutualism, commensalism and parasitism (Prins 1975). Some of the interactions that have been observed in the rumen are discussed below.

1.7.1 Protozoal interactions

1.7.1.1 Protozoa-bacteria interactions

The most obvious interaction between the ruminal ciliate and bacteria is a predator-prey relationship, although symbioses and parasitism have been exemplified (Williams

et al. 1993, Williams and Lloyd 1993). Gutierrez and workers first recognized predation of rumen bacteria by rumen ciliate protozoa (Gutierrez 1955, Gutierrez and Hungate 1957, Gutierrez 1959). Predation does not appear to be species-specific and is more a function of bacterial concentration. Bacteria attached to fiber, i.e. cellulolytic and hemicellulolytic species are an exception as they may be less likely ingested by protozoa (Prins 1991). Bacteria constitute the main source of nitrogenous compounds for protozoal growth. The proportions of bacterial species present and the overall size of the bacterial population can be 50-90% lower in faunated animals than in ciliate free animals (Williams and Coleman 1997). Defaunation improves the efficiency of net microbial synthesis in the rumen and increases the flow of microbial protein to the small intestines (Jouany 1989). Axenic bacterial-free cultures of rumen protozoa have not been established and so the protozoa must be considered as parasitic. The bacteria can function in the absence of protozoa, but the converse does not appear to be true (Dehority 1998).

1.7.1.2 Protozoa-protozoa interactions

The best-known interaction between rumen protozoa is predation of one ciliate species by another (Eadie 1962, Eadie 1967). Predation by species of *Diplodinium, Elytroplastron, Enoploplastron, Entodinium, Metadinium, Ostracodinium,* and *Polyplastron* on other rumen ciliates and cannibalistic predation by *P. multivesiculatum* have been reported (Williams and Coleman 1992). *Polyplastron* requires the presence of *Epidinium* for growth *in vitro,* as it appears to be a food source (Coleman 1972, Coleman and J. 1978). They are engulfed and can be replaced by species of *Diplodiniinae* but not *Entodinia*. *Entodinium bursa* also has an obligate requirement for the spineless form of *Entodinium caudatum.* The engulfed protozoa are digested and are an important source of protein (Williams and Coleman 1992).

1.7.1.3 Protozoa-fungi interactions

The evidence for predation of fungi by protozoa in co-cultures is variable. Defaunation either led to increase (Orpin 1977, Soetanto et al. 1985, Romulo et al. 1986) or no significant effect on fungal densities (Jouany 1989, Williams and Withers 1991, Williams and Withers 1993). Ruminal ciliates reduced *N. patriciarum* carboxymethylcelluase activity, while the cellulolytic activity per unit biomass of *Piromyces* sp. increased in the presence of ruminal ciliates (Morgavi et al. 1994, Widyastuti et al. 1995). In another study, combined cell-free lysates of *N. frontalis* and individual ciliates exhibited enhanced hemicellulolytic activity, whereas the activities of some cellulolytic enzymes were reduced in the presence of *Entodinium* spp. (Williams et al. 1993). However, studies *in vitro* have confirmed protozoal predation of fungal rhizoids (Joblin 1990). Scanning electron microscopy also clearly showed that *Polyplastron multivesiculatum, Eudiplodinium maggii* and *Entodinium* spp. are able to ingest fungal rhizoids and sporangia (Williams and Coleman 1992, Williams et al. 1993, Williams and Lloyd 1993, Williams et al. 1994).

1.7 2 Bacterial interactions

1.7.2.1 Bacteria-fungi interactions

Anaerobic fungi, through their penetration and degradation of plant tissue and their production of hydrogen (H_2), may actively shape the remainder of the microbial community, such as bacteria, archaea, and ciliate protozoa. Methanogens in turn use the H_2 produced by fungi, and stable co-cultures of fungi and methanogens have been successfully established *in vitro* (Bauchop and Mountfort 1981, Mountfort et al. 1982, Fonty et al. 1988). Fungi are also involved in cross feeding by releasing sugars and other metabolites, which serve as energy sources for bacterial species. They may also

depend on bacteria to supply their nutritional requirements of B vitamins and amino acids (Williams et al. 1994, Theodorou et al. 1996).

In addition, although anaerobic fungi are very actively cellulolytic when grown in pure culture, they have been shown to be less active in mixed cultures with cellulolytic bacteria. Some cellulolytic bacteria including strains of *R. flavefaciens* and *R. albus* have been shown to produce soluble proteins that inhibit cellulolysis by growing fungi (Stewart et al. 1992, Bernalier et al. 1993). A chitinolytic bacterium similar to *C. tertium* has also been reported to inhibit cellulolysis in several types of anaerobic fungi by expressing chitinases (Kopecny et al. 1996).

1.7.2.2 Bacteria-Bacteria interactions

Interactions between bacterial species can be positive i.e. synergism and cross-feeding or negative, i.e. production of compounds inhibitory to other bacterial species (Dehority 1998). Positive interactions, which have been observed, include the marked synergism in digestion of structural carbohydrates (Dehority and Scott 1967). Studies on the major cellulolytic species, *F. succinogenes, R. albus* and *R. flavefaciens* show that they effectively digest cellulose alone with no increases observed when any two were combined in the same fermentation. However, when the non-cellulolytic organism *Prev. ruminicola* was combined with any of the cellulolytic species, digestion was increased (Dehority and Scott 1967). Synergism between *F. succinogenes* and *Prev. ruminicola* enhanced activity against purified pectin (38% increase), whereas, neither of these organisms have previously shown much activity against pectin (Osborne and Dehority 1989). Combining a hemicellulose degrading but non-utilizing cellulolytic species with a non-degrading but utilizing hemicellulolytic species results in extensive hemicellulose digestion from intact forage (Coen and Dehority 1970, Morris and Vangylswyk 1980, Osborne and Dehority 1989, Dehority 1993). On the other hand, it has also been

observed that some co-culture combinations reduced the extent of digestion. Decreases in forage degradation have been observed between *F. succinogenes* and *R. flavefaciens* (Saluzzi et al. 1993) when simultaneously present in a fermentation medium and between *R. albus* and *R. flavefaciens* (Odenyo et al. 1994).

Other positive interactions include cross feeding of hydrolysis products, utilization of end products and essential nutrients. For example, non-cellulolytic bacteria can utilize cello- dextrins produced by cellulolytic species (Russell 1985). Increase in xylan utilization has been observed when *R. flavefaciens* was co-cultured with *Met. smithii* (Williams et al. 1994). Production of nutrients by one species, which is essential for the growth of another species also occurs in the rumen (Wolin et al. 1997).

1.8 Introduction to antimicrobial peptides

Antimicrobial peptides (AMPs) are an integral part of the innate host defence system of many organisms including vertebrates, invertebrates, plants and bacteria (Wiesner and Vilcinskas 2010), with broad spectrum activity against several groups of organisms including bacteria, fungi, viruses and parasites (Jenssen et al. 2006). Due to this, AMPs represent one of the most promising new sources of antibiotics and future strategies for defeating the threat of antimicrobial resistance in bacterial infections (Hancock 1997). Bacteriocins (briefly discussed in section 1.9) were among the first antimicrobial peptides to be isolated and characterized (Gratia 1925). This was followed by the discovery of gramicidins and polymyxins (including Polymyxin B and Colistin) in 1939 and 1960 respectively (Dubos 1939, Dubos 1939, Wright and Welch 1960). These early antimicrobial peptides tended to be cytotoxic and the availability of alternative agents against pneumococci infections limited their usage as injectables (Hancock 1997). AMPs have also been referred to as anionic antimicrobial proteins/peptides, cationic host defence peptides, cationic amphipathic peptides, α-helical antimicrobial peptides

and cationic AMPs (Groenink et al. 1999, Bradshaw 2003, Brown and Hancock 2006, Harris et al. 2009, Huang et al. 2010, Riedl et al. 2011). Many AMPs have been discovered or synthesized to date. There are 2544 (250 bacteriocins from bacteria, 2 from archaea, 7 from protists, 13 from fungi, 317 from plants, and 1915 from animals) and 5547 AMPs (3904 natural and 1643 synthetic) in the antimicrobial peptide database (APD2) and LAMP- a database linking antimicrobial peptides (Wang et al. 2009, Zhao et al. 2013). The biophysical and structural characteristics of AMPs play an important role in their activity against pathogenic bacteria. These include secondary structure, charge, amphipathicity and hydrophobicity. The individual importance of these parameters are difficult to determine as they all influence each other, however, some general trends have emerged from studies with model peptides (Zhao 2003, Cirac 2011). Antibacterial peptides are the most studied AMPs to date and most of them are cationic AMPs (Bahar and Ren 2013).

Cationic AMPs are generally defined as peptides of up to 50 and less than 100 amino acids in residues with a net positive charge of +2 to +9 at neutral pH owing to the presence of multiple basic amino acids, lysine (K) and arginine (R) (and of modified amino acid residues for the bacterially produced lantibiotics and polymyxins) over acidic residues and a substantial portion (≥30% or more) of hydrophilic amino acid residues (Hancock 1997, Hancock and Chapple 1999, Powers and Hancock 2003, Giuliani et al. 2007). Another common trait shared by cationic peptides is their ability to fold into either amphipathic or amphiphilic conformations (have both a hydrophobic face comprising non-polar amino acid side chains and a hydrophilic face of polar and positively charged residues), induced by interaction with bacterial membranes or membrane mimics (Powers and Hancock 2003). Cationic peptides not only have antimicrobial activities against Gram positive and Gram negative bacteria but have also

been shown to have activity against fungi, protozoa, viruses, and have anticancer and immunomodulatory properties (Baker et al. 1993, Aley et al. 1994, Mohammad et al. 1995, Scott et al. 1999, Johnstone et al. 2000, Wong et al. 2013).

Cationic peptides rarely have the exceptionally high *in vitro* antimicrobial activities demonstrated with some conventional antibiotics against selected bacteria (Piers et al. 1994, Gough et al. 1996). Some AMPs do however, have minimum inhibitory concentrations (MIC) in the range 1-8 µg/mL that are competitive with those found for even the most potent antibiotics against resistant organisms (such as *P. aeruginosa, A. baumanii*, *Stenotrophomonas maltophilia,* methicillin resistant *S. aureus*, vancomycin-resistant enterococci, extended spectrum 6-lactamase-producing *E. coli*, and multiple-antibiotic-resistant Gram negative bacteria (Hancock 1997). These peptides, at concentrations around the MIC, kill bacteria much more quickly than conventional antibiotics do, an observation that has been ascribed to their mode of action. Some naturally resistant bacteria e.g. *Burkholderia cepacia* and *Serratia marcescens* exist by virtue of a non-interactive outer membrane and production of specific proteases, respectively (Hancock 1997). However, AMP MICs against clinically antibiotic resistant and clinically susceptible strains of a given species do not vary greatly and most cationic peptides do not induce resistant mutants even after as many as 20 passages on an antibiotic concentration close to the MIC (Hancock 1997).

1.8.1 Mode of action of cationic AMPs

Due to the renewed interest in AMPs as therapeutic alternatives, the mode of action of AMPs is extensively being studied. These studies have indicated that all antimicrobial peptides interact with membranes and tend to divide peptides into two mechanistic classes: membrane disruptive and non-membrane disruptive (Hwang and Vogel 1998, Oren and Shai 1998, Epand and Vogel 1999, Hancock and Chapple 1999, Shai 1999,

Hancock and Rozek 2002, Zasloff 2002, Huang 2006). However, there is evidence that AMPs can act in alternative ways by inhibiting several cellular processes such as cell wall synthesis, enzymatic activity, macromolecular synthesis (nucleic acid and protein synthesis) and that the action responsible for killing bacteria at the minimal effective concentration varies from peptide to peptide and from bacterium to bacterium (Lehrer et al. 1989, Yonezawa et al. 1992, Kragol et al. 2001, Patrzykat et al. 2002, Yeaman and Yount 2003, Brogden 2005, Hancock and Sahl 2006, Jenssen et al. 2006, Nicolas 2009).

In order to explain the mechanism of action of AMPs against bacteria, the morphology of the bacterial cell must be taken into account. The rigid structure of the bacterial cell wall provides strength and shape and protects the cell from changes in its environment. The differences in the composition of bacterial cell wall also determines mode of action of AMPs (Figure 1.11 illustrates the molecular basis of cell selectivity in different cell types). Whereas, Gram positive bacteria cell wall is composed of a peptidoglycan layer and a single cytoplasmic bilayer, the Gram negative cell wall is composed of two bilayers, an outer negatively charged lipopolysaccharide (LPS) -phospholipid molecule bilayer stabilized by divalent cations like Ca^{2+} and Mg^{2+} and an inner single cytoplasmic membrane. One of the most interesting properties of AMPs is their cell selectivity for prokaryotic over mammalian membranes. Lower affinity of AMPs can be explained by the zwitterionic (neutral) phospholipids which make up outer membranes of mammalian cells (Matsuzaki 2009, Melo et al. 2009). The strong presence of membrane stabilizing sterols also protects mammalian cells and makes them less susceptible to AMPs (Mason et al. 2007, Matsuzaki 2009).

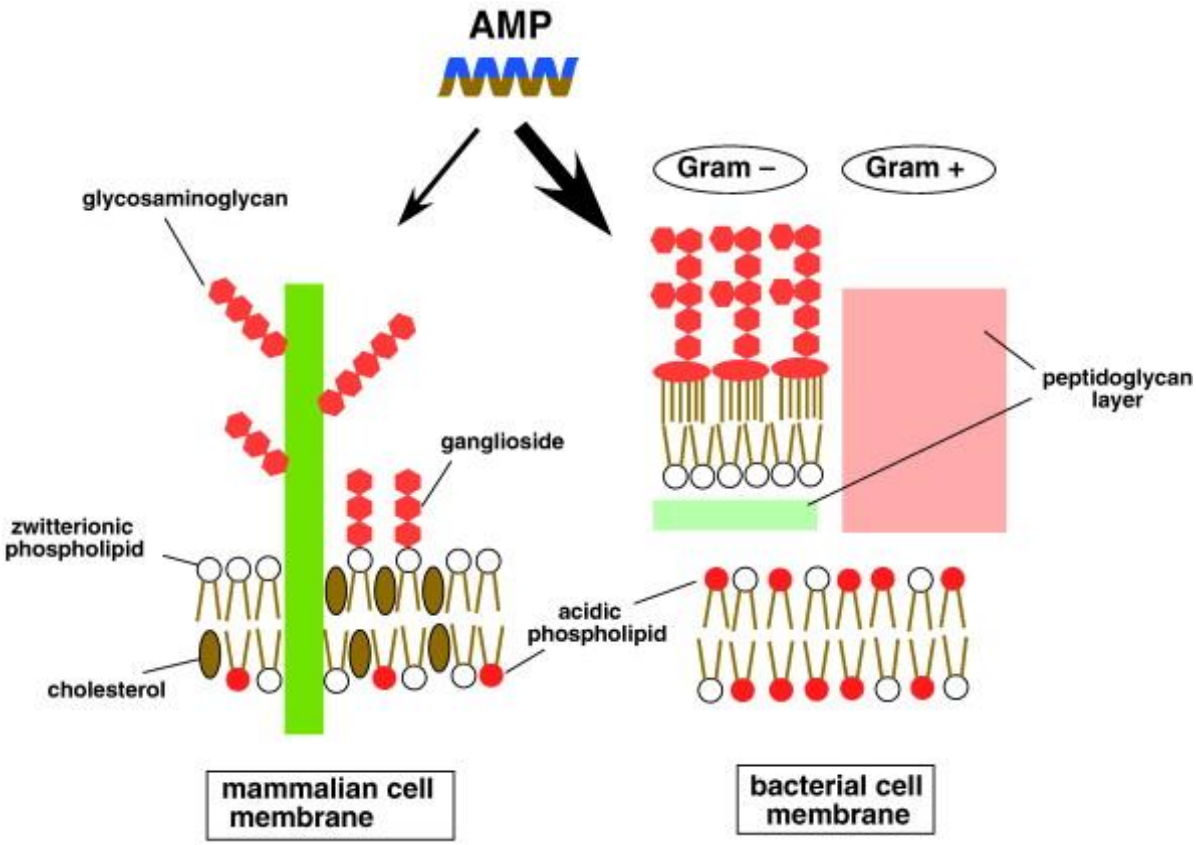


Figure 1.11 Molecular basis of cell selectivity of AMPs. AMPs form amphipathic structures with a positively charged face (blue) and a hydrophobic face (brown). Electrostatic interaction between the positive charges of AMPs and negatively charged components (red) at the mammalian cell surface (left) and bacterial surface (right) is the major driving force for cellular association. Source: (Matsuzaki 2009).

The mechanism of action against Gram negatives is better studied. The initial association of peptides with the bacterial membranes occurs through electrostatic interactions between the cationic peptide and the anionic LPS in the outer membrane of Gram negative bacteria and wall-associated teichoic acids in Gram positive bacteria leading to perturbation of the membrane. Since cationic peptides have higher affinity for the LPS than divalent cations (Hancock and Chapple 1999), cationic peptides displace these divalent cations and cause a local disturbance in the outer membrane, which facilitates the formation of destabilized areas through which the peptide translocates the outer membrane in a process termed self-promoted uptake (Hancock 1997, Hancock 2001) (Figure 1.12). The peptides then associate with the outer monolayer of the

cytoplasmic membrane. It is at this point that membrane disruptive and non-membrane disruptive mechanisms diverge, depending on whether this reorientation leads to perturbation of the integrity of the cytoplasmic membrane or peptide translocation into the cytoplasm (Powers and Hancock 2003). Membrane disruptive peptides are generally reported to be of the α-helical structural class although, not all α-helical peptides are membrane disruptive (Park et al. 1998, Friedrich et al. 2001, Patrzykat et al. 2002).

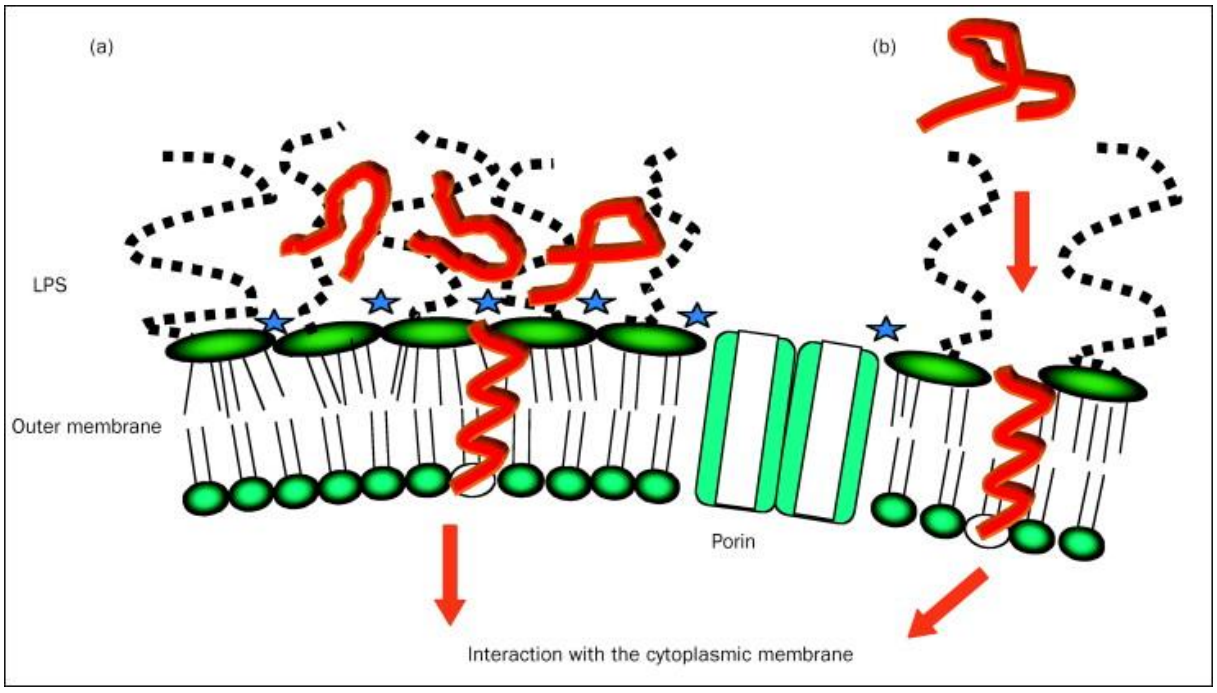


Figure 1.12 Self-promoted uptake of cationic peptides across the outer membrane. Unfolded cationic peptides are proposed to associate with the negatively charged (mainly due to the presence of highly anionic lipopolysaccharide [LPS]) surface of the outer membrane. They then either neutralise the charge over a patch of outer membrane, creating cracks through which the peptide can cross the outer membrane (a) or actually bind to the divalent cation binding sites on lipopolysaccharide, and disrupt the membrane (b). Once the peptide has crossed the outer membrane, it will interact with the negatively charged surface of the cytoplasmic membrane. Source: (Hancock 2001).

Several AMP membrane interaction and disruption models have been developed to explain membrane disruption. The most prominent mechanistic models include the "barrel stave", "carpet" models, and "micellar aggregate (toroidal models)" (Figure 1.13).

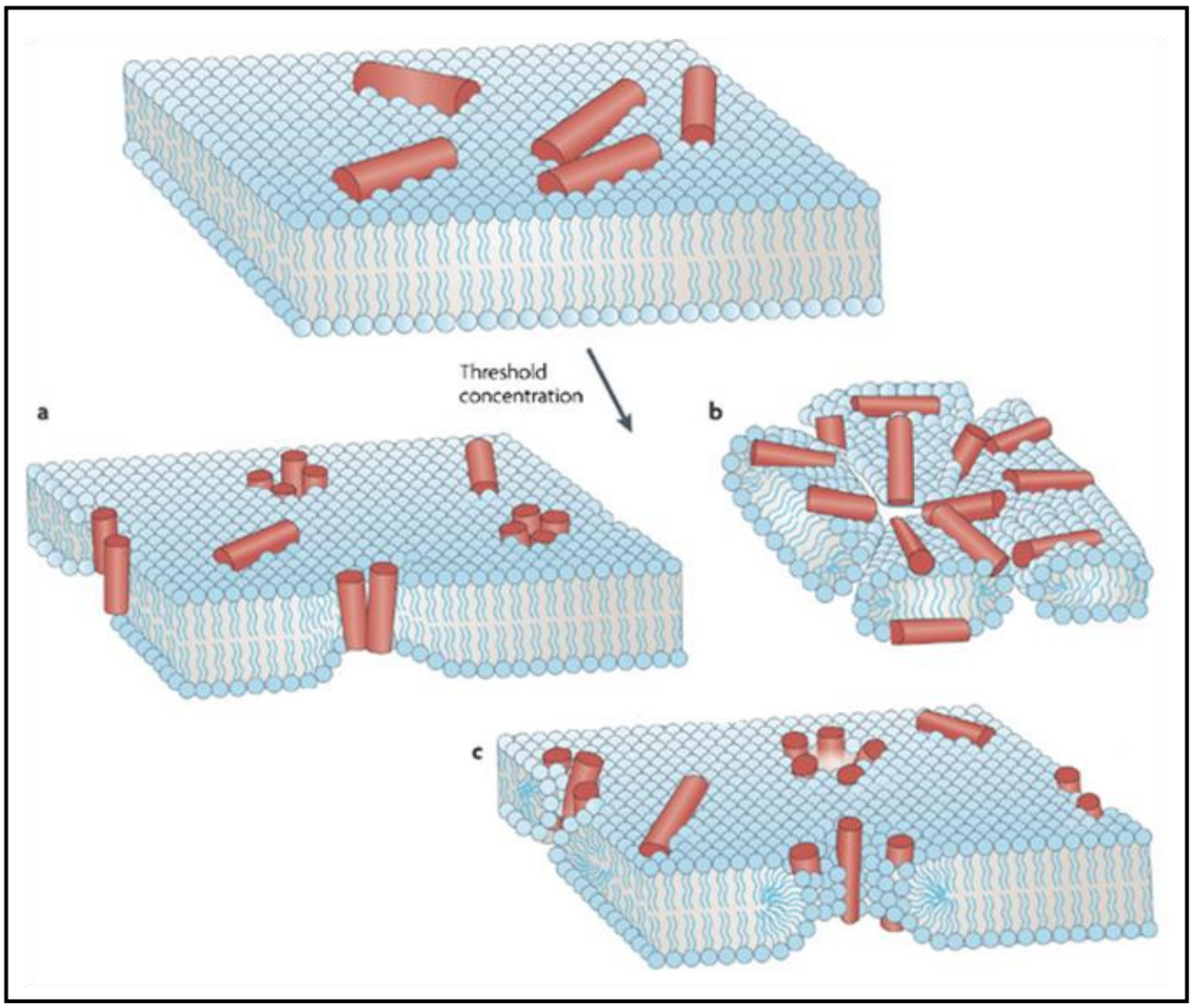


Figure 1.13 Mechanisms of AMP-mediated membrane disruption (a) barel-stave model, (b) carpet model (c) micellar aggregate (toroidal pore) model. Source: (Melo et al. 2009).

1.8.1.1 Barrel-stave model

In the barrel-stave model, the amphipathic peptides reorient perpendicular to the membrane and align (like the staves in a barrel) in a manner in which the hydrophobic sidechains face outwards into the lipid environment while the polar sidechains align inward to form transmembrane pores. Progressive recruitment of additional peptide monomers leads to a steadily increasing pore size proposed to allow leakage of cytoplasmic components and also disrupt the membrane potential (Ehrenstein and Lecar 1977). This pore formation requires peptides to be sufficiently long to traverse the

hydrophobic core of the membrane without inserting the polar groups of the lipid. Pardaxin, alamethicin and Gramicidin S have been shown to kill bacteria using this model (Rapaport and Shai 1991, Sansom 1993, Yang et al. 2001, Zhang et al. 2001). The major argument against this model is the lack of preferred stoichiometries for the "pores" as demonstrated by the wide variability in conductance increases induced by peptides in model membranes (Wu et al. 1999).

1.8.1.2 Carpet model

In the alternative carpet model (Pouny et al. 1992), the peptides do not insert into the membrane but align parallel to the bilayer through a relatively diffuse manner ('carpet-like' cluster of peptides), remaining in contact with the lipid head groups and effectively coating the surrounding area. This orientation leads to a local disturbance in membrane stability, causing the formation of large cracks, leakage of cytoplasmic components, disruption of the membrane potential and, ultimately, disintegration of the membrane (breakage into pieces and lysis of the microbial cell when a threshold concentration of peptide is reached) (Oren and Shai 1998). This model has been used to describe the mode of action of antimicrobial peptides, such as dermaseptin and its natural analogues, cecropins, and the human antimicrobial peptide LL-37 (Pouny et al. 1992, Gazit et al. 1995, Ghosh et al. 1997, Oren et al. 1999).

1.8.1.3 Micellar aggregate model

The alternative micellar aggregate model (Matsuzaki et al. 1997, Hancock and Chapple 1999) suggests that the peptides reorient and associate in an informal membrane-spanning micellar or aggregate-like arrangement and further indicates that collapse of these micellar aggregates can explain translocation into the cytoplasm. After binding to the phospholipid head groups, the peptides insert into the membrane and then cluster

into unstructured aggregates that span the membrane. These aggregates are proposed to have water molecules associated with them providing channels for leakage of ions and possibly larger molecules through the membrane. This model essentially differs from the other two, in that, only short-lived transmembrane clusters of an undefined nature are formed (informal channels with a variety of sizes), which allow the peptides to cross the membrane without causing significant membrane depolarization, and once inside the cell, the peptides exert their killing activities on intracellular targets. AMPs melittin, magainin and LL-37 have been proposed to exert this mechanism (Brogden 2005, Jenssen et al. 2006, Leontiadou et al. 2006, Sengupta et al. 2008).

Regardless of which model is correct, the net result of membrane disruption would be the rapid depolarization of the bacterial cell leading to rapid cell death, with total killing occurring within 5 minutes for the most active peptides (Friedrich et al. 1999). Membrane depolarization however, is not a lethal event per se, and in the absence of evidence of a catastrophic collapse of cytoplasmic membrane integrity, the specific way in which membrane disruption results in cell death is yet to be determined (Powers and Hancock 2003). Any of the above models might be correct depending on the peptide examined, such that certain peptides may function through a barrel-stave mechanism, while others may function through a micellar aggregate or carpet mechanism.

1.8.2 Classification of cationic AMPs

The vast diversity of these AMPs which arise from their antimicrobial function and the different pathogenic microbe challenges they face in each host organism (Hancock and Sahl 2006) makes it difficult to classify them. They can be classified according to their biosynthesis pathway (as non-ribosomally synthesised (Mootz and Marahiel 1997, Konz and Marahiel 1999) and ribosomally synthesised peptides (Nissen-Meyer and Nes

1997)). They can also be classified according to their secondary structures, source, type of and predominant amino acid residues (as defensins, histatins, cathelicidins lantibiotics, and peptaibols (Klaenhammer 1993, Franklin et al. 1994, Van Den Hooven et al. 1996, Hwang and Vogel 1998, Ganz 2003, Zanetti 2004)). For ease, they are broadly classified into four major groups: α-helical, β-sheet, loop and extended peptides based on their secondary structures (Koradi et al. 1996, Hancock and Lehrer 1998) (Figure 1.14). Several synthetic variant peptides which fall into these structural classes have also been produced.

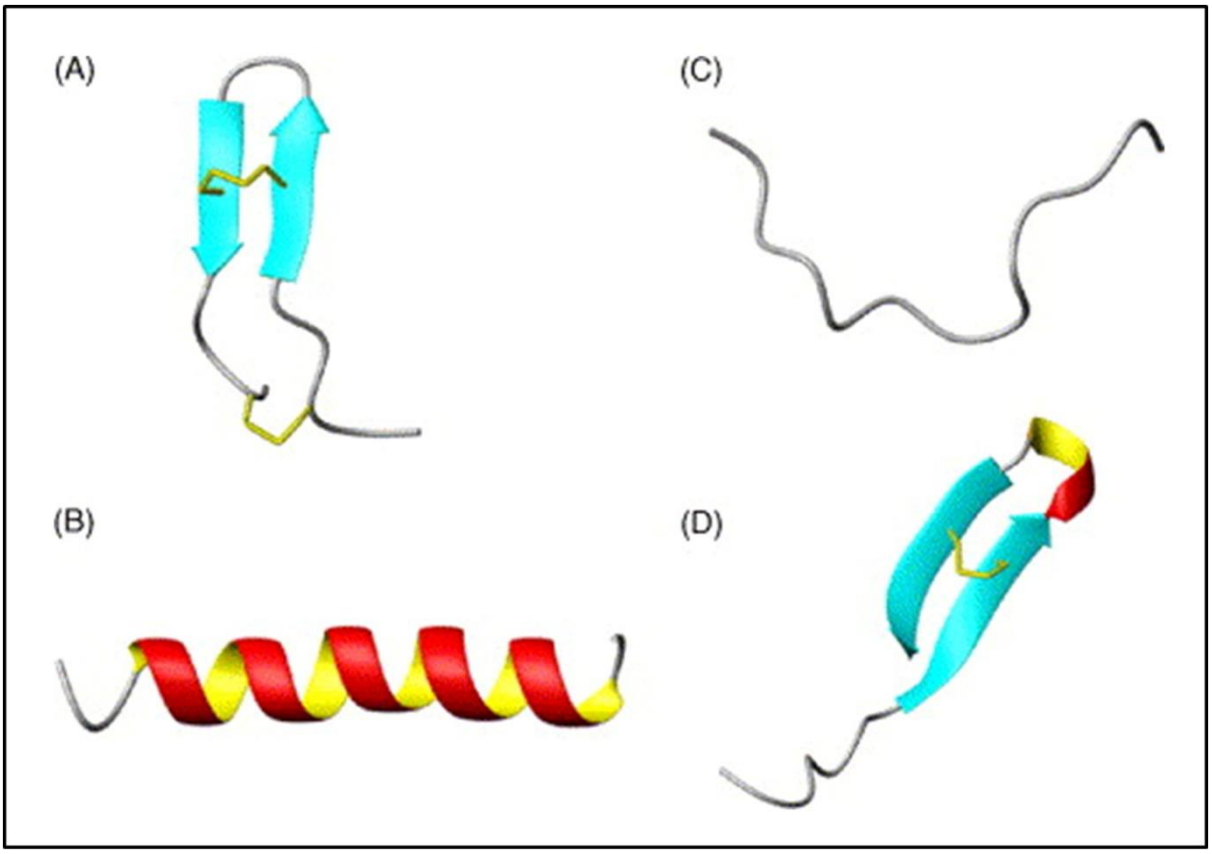


Figure 1.14 Structural classes of antimicrobial peptides: (A) β-sheet, (B) α-helical, (C) extended, (D) loop. Disulfide bonds are indicated in yellow. Source: (Powers and Hancock 2003)).

1.8.3 Structure function relationships of AMPs

Peptides undergo significant conformation changes upon binding to the target cell. The importance of these secondary structures (conformations) in the activity of AMPs has been extensively studied in order to extract a structure-function relationship.

1.8.3.1 α-helical peptides

Linear AMPs with an α-helical structure (Fig 1.14 B) are one of the larger and better studied classes of AMPs. These AMPs tend to be disordered and are highly flexible in aqueous solution. They are characterized by their α-helical conformation upon interaction with membranes and membrane-mimicking environments (Powers and Hancock 2003). They are often found to be amphipathic and can either absorb onto membrane surfaces or insert into the membrane as a cluster of helical bundles. They may also contain a bend in the centre of the molecule which may be critical for selectivity by suppressing haemolytic activity (Zhang et al. 1999).

Magainins (1 and 2) are one of the most studied AMPs of the α-helical class. The 23 amino acid peptides were first isolated from the African clawed frog *Xenopus laevis*, and possess modest antimicrobial activities (50 μg/ml) against *E. coli* (Zasloff 1987). The structure of magainin 2 was determined by Proton Nuclear Magnetic Resonance (^{1}H NMR) Spectroscopy in the presence of dodecylphosphocholine (DPC), and sodium dodecylsulfate (SDS) micelles and was shown to adopt an amphipathic α-helical conformation with a slight bend centred at residues 12 and 13 (Gesell et al. 1997). Its antimicrobial mechanism has been proposed to involve selective bacterial membrane permeabilization leading to disruption of membrane potential (Matsuzaki 1998). This mechanism is supported by the observation that no difference exists between D- and L enatiometric peptides, ruling out the involvement of a chiral receptor or an enzyme as a

target (Bessalle et al. 1990, Wade et al. 1990). The mechanism of action has been shown to follow the micellar-aggregate model of antimicrobial activity where magainins interact with negatively charged phospholipids and spontaneously form transient, membrane spanning pores, which, upon collapse, permit peptide translocation to the inner leaflet (Matsuzaki et al. 1995, Matsuzaki et al. 1997). Membrane disruption (Matsuzaki et al. 1989, Matsuzaki et al. 1991, Matsuzaki et al. 1995) and magainin induced depolarization has also been demonstrated in model in *E. coli* as well as in and model systems (Juretic et al. 1989, Juretic et al. 1994). N-Terminal truncation of α-helical magainin 2 indicates that truncation of the peptide to fewer than 20 residues (i.e. deletion of residue 4 and above) results in a compound that is unable to span the lipid bilayer and thus, from a mechanistic perspective, explains the corresponding loss of antimicrobial activity (Zasloff et al. 1988). However, α-helical peptides with as few as 13 residues can possess antimicrobial activity so an ability to span a lipid bilayer is not an obligate requirement for activity of α-helical peptides (Zhang et al. 2001).

In both the membrane-disruptive and non-membrane-disruptive mechanisms of peptide antimicrobial activity, the initial step is the interaction of the cationic peptide with the negatively charged cell surface. Therefore, to determine the forces leading to favourable association and ascertain if this step is simply driven by electrostatic attraction, the contribution of charge toward the activity of magainin 2 was investigated using analogues with varying cationic charges (Dathe et al. 2001). It was observed that charge increase to +5 is accompanied by a corresponding increase in antimicrobial activity, while further increase of charge to +7 did not alter the maximal activity observed at +5, but increased, haemolytic activity. Interestingly, experiments using model membranes composed of the anionic lipid phosphatidylglycerol found that an increase in charge actually led to a decrease in membrane permeabilizing ability. This is likely a result of

the corresponding decrease in hydrophobicity that accompanies an increase in charge (Dathe et al. 2001).

1.8.3.2 β-sheet peptides

In contrast to linear α-helical peptides, β-sheet peptides (Figure 1.14 A) have a more restrained conformation due to their cyclic structure formed either by disulfide bonds as in the case of human β-defensins tachyplesins, lactoferricin and protegrins (Nakamura et al. 1988, Jones et al. 1994, Harwig et al. 1995, Matsuzaki 1999, Hancock 2001) or by cyclization of the peptide backbone as in the case of gramicidin S, polymyxin B and tyrocidines (Prenner et al. 1999, Zaltash et al. 2000, Bu et al. 2002). They are largely characterized by the presence of an antiparallel β-sheet in aqueous solution that may be further stabilized upon interactions with lipid surfaces. Larger peptides within this peptide class may also contain small helical segments (Powers and Hancock 2003).

Tachyplesins are the best characterized β-sheet peptides. Originally isolated from the haemocytes of the Japanese horseshoe crab, *Tachypleus tridentatus*, and with only 17–18 residues (Nakamura et al. 1988), they represent a convenient scaffold for structure–activity studies due to their small size and availability of a high-resolution ^{1}H NMR structure. Tachyplesin I is an antiparallel β-sheet (residues 3–8 and 11–16) connected by a type I β-turn (residues 8–11), stabilized by two disulfide bonds (residues 3 and 16 and residues 7 and 12) with an amidated C-terminus (Kawano et al. 1990). It has moderate antimicrobial activity (<12.5 μg/ml MIC) against *E. coli* K12 (Nakamura et al. 1988) and a high affinity for lipopolysaccharides (Hirakura et al. 2002).

Although the structure and *in vitro* activity of this group of peptides especially tachyplesins are well characterized, the exact mechanism of antimicrobial activity remains poorly understood. Whereas tachyplesins have a high affinity for LPS, they also have intracellular targets and have been shown to bind DNA (Yonezawa et al.

1992) and are effective at inducing lipid flip-flop and undergoing membrane translocation but do not cause substantial calcein release from model membrane systems (Zhang et al. 2001). This suggests these peptides disrupt lipid organization leading to the translocation of peptide molecules across the bilayer but do not form long-lived pores or channels (transient pore formation) (Matsuzaki et al. 1997). Thus, these peptides may function through a micellar-aggregate or related model of translocation as seen in the α-helical magainin.

Several structure activity relationships (SAR), to understand the importance of disulfide bonds in the antimicrobial activity of this group of peptides, have been carried out. Generally, linearization and replacement of cysteine residues with certain amino acids like Alanine (A), Aspartic acid (D) and Leucine (L), lead to reduced antimicrobial activity or inactivation of the peptides as well as reduced calcein release from model membranes (Matsuzaki et al. 1993, Tamamura et al. 1993, Matsuzaki et al. 1997, Rao 1999) while analogues with aromatic and hydrophobic residues retained broad spectrum activity (Tamamura et al. 1998). These studies suggest that the cyclic structure is essential for antimicrobial activity while it may not be crucial for membrane permeabilization (Matsuzaki et al. 1997, Tamamura et al. 1998, Rao 1999). Moreover, major differences in solution and micelle-bound structures of tachyplesin and a linear analogue analysed by ^{1}H NMR highlight the need for high-resolution peptide structures, rather than simple conformational analyses by circular dichroism, to provide detailed structure–activity information (Laederach et al. 2002).

1.8.3.3 Extended peptides

Linear peptides with an extended structure (Figure 1.14 C) lack classical secondary structures (generally due to their high proline (P) or glycine (G) contents) and are characterized by overrepresentation of specific amino acids. For example, Histatin

produced in saliva is highly rich in histidine (H) residues (Tsai and Bobek 1998) and indolicidin isolated from the cytoplasmic granules of bovine neutrophils is highly rich in tryptophan (W) residues (Selsted et al. 1992). These extended peptides form their final structures by hydrogen bond and Van der Waals interactions with membranes instead of through interresidue hydrogen bonds (Powers and Hancock 2003).

Indolicidin, a 13-residue, C-terminal amidated peptide is the best characterized representative of this group of peptides. It has the highest known proportion of tryptophans (5 out of 13 residues) in a peptide and its conformation is dependent on its environment (Selsted et al. 1992). It possesses reasonable antimicrobial activity (MIC of 10 μg/ml) against *E. coli* but a lower affinity for LPS compared to other peptides such as the β-hairpin tachyplesins (Falla et al. 1996, Hirakura et al. 2002). The structure of indolicidin has been determined by ^{1}H NMR in both anionic SDS and zwitterionic dodecylphosphocholine (DPC) micelles and it exists in an extended conformation in both lipid environments, but takes a more bent conformation due to two half-turns about residues 5 and 8 in neutral DPC micelles (Rozek et al. 2000).

It has been hypothesized that indolicidin acts by disrupting the cytoplasmic membrane of *E. coli* by voltage-induced channel formation driven by membrane potential (Falla et al. 1996, Subbalakshmi and Sitaram 1998, Wu et al. 1999) as its size (25×32 Å) makes it possible to span biological membranes (Selsted et al. 1992). In other studies however, indolicidin was unable to completely depolarize the cytoplasmic membrane of *E. coli* and *S. aureus* (Wu et al. 1999, Friedrich et al. 2000) arguing against membrane disruption as a mechanism. The peptide has also been shown to induce filamentation of *E. coli*, which is thought to be a result of DNA synthesis inhibition (Subbalakshmi and Sitaram 1998). In order for this mechanism to be effective, membrane translocation must obviously occur. Therefore, in accordance with the micellar-aggregate model of

antimicrobial activity, both hypotheses combine to explain the actions of indolicidin; the formation of informal aggregate channels that, upon collapse, lead to translocation of the peptide into the cytoplasm (Powers and Hancock 2003).

Several indolicidin analogues (including CP-11 and CP10A) have been synthesized to understand the importance of tryptophan and proline residues and the charge requirements for antimicrobial activity (Falla et al. 1996, Subbalakshmi et al. 1996, Zhang et al. 2001). It appears to be conformational changes rather than changes in charge or hydrophobicity that account for differences in activity in the case of the indolicidin family of peptides. For example, change in conformation from extended to helical in the analogue CP10A, led to increased membrane insertion and improved membrane translocation, allowing better access to the cytoplasm and cytoplasmic target (Friedrich et al. 2001).

1.8.3.4 Loop peptides

The last class of cationic peptides to be outlined in this thesis are the loop peptides (Fig 1.14 D). They are made up of lantibiotics e.g. nisin and mersacidin, and peptides with loop structures imparted by the presence of a single bond (disulfide, amide or isopeptide), e.g. thanatin, lactoferricin B and bactenecin-1. Thanatin, a 21-residue, loop peptide isolated from the spined soldier bug, *Podisus maculiventris* (Fehlbaum et al. 1996) is the only member of this group with an available high-resolution solution structure determined by ^{1}H NMR. It is an anti-parallel β-sheet, formed by residues 8–21, stabilized by the single disulfide bond between residues 11 and 18 (Mandard et al. 1998). The peptide has reasonable antimicrobial activity against Gram-negative and Gram positive bacteria as well as fungi (Fehlbaum et al. 1996) and is comparable in activity to members of the β-sheet family of peptides.

Although, the exact antimicrobial mechanism of thanatin remains unknown, it is thought to act on targets other than the membranes, as treatment with peptide does not induce changes in permeability and mechanism of killing is believed to be dependent on the organism (Fehlbaum et al. 1996). Both D- and L-enantiomers are equally active against Gram-positive and fungal species but only L-thanatin is active against Gram-negative bacteria, thus suggesting that a stereospecific target such as a receptor may be involved in Gram-negative bacteria while non-specific interactions dominate in both fungi and Gram-positive bacteria (Fehlbaum et al. 1996). Truncation of the C-terminus or beyond the third N-terminal residue has also been shown to greatly reduce activity as the loop region alone is completely inactive (Fehlbaum et al. 1996).

1.8.4 Therapeutic potential of AMPs

The considerations that will determine any clinical use of cationic peptides include toxicity, stability, immunogenicity, route of application, and formulation (Hancock 1997). Cationic peptides have several assets that make them excellent prospects as novel antimicrobial agents. In addition to their microbicidal activity, they also possess other biological activities and have potential applications as signalling molecules, immune modulators, antitumor agents, drug delivery vehicles and plant transgenesis mediators (Zhang and Falla 2010, Pushpanathan et al. 2013, Otvos and Ostorhazi 2015, Wang et al. 2015). There is also potential for AMP use as topical therapy and wound healing (Otvos and Ostorhazi 2015). Such treatments are likely to be effective and safe because the more toxic cationic peptides and lipopeptides, including gramicidin S and polymyxin B, are already in skin creams. Unlike most antibiotics, at least some cationic peptides neutralise LPS and prevent endotoxemia during cell killing and/or lysis (Gough et al. 1996). This ability to prevent endotoxinaemia, which not all cationic peptides possess, is a great advantage for these peptides over other antibacterial agents. AMPs

have also been shown to possess “enhancer” activity (ie, synergy with classical antibiotics) (Corrigan and Bell 1971, Hancock et al. 1995). The more resistant an isolate is to a given antibiotic, the more profound is the enhancement of activity by an appropriate cationic peptide. Thus cationic peptides have the ability to serve as antiresistance compounds. Cationic peptides with excellent activity against a broad range of bacteria would be especially indicated where there is a risk from seriously resistant pathogens or where current treatments are ineffective.

Intense research efforts over the years to overcome the hurdle of AMP use, have led to significant advances including optimizing peptide activity and stability under physiological conditions, lowering toxicity, deciphering the role of AMPs in modulating the immune system and how this can be exploited for therapeutic benefit. Therefore, despite some initial draw backs, (high cost of production, unwanted side effects, insufficient stability and activity under physiological conditions and upcoming adaptive bacterial resistance), which hindered cationic AMP use as therapeutics, more cationic peptides now seem to enter clinical trials (Table 1.6 below), indicating some success in developing peptides into therapeutics (Fox 2013, Ashby et al. 2014, Strempel et al. 2015). Nonetheless, a knowledge gap still exists and continuous efforts into peptide-based drug design is encouraged if these bottlenecks are to be completely overcome.

Table 1.6 Antimicrobial peptides in clinical development. (Adapted from (Gordon et al. 2005, Fjell et al. 2012, Fox 2013, Ashby et al. 2014)).

Product	Description	Indication	Clinical trial phase	Company (location)
Magainin peptide/pexiganan acetate	22-amino-acid linear antimicrobial peptide, isolated from the skin of the African clawed frog (*Xenopus laevis*)	Diabetic foot ulcers	III (complete)	Dipexium Pharma (White Plains, New York)/MacroChem/Genaera) (formerly Magainin Pharmaceutical Inc.)
Omiganan	Synthetic cationic peptide derived from bovine indolicidin	Rosacea	III (complete)	BioWest Therapeutics/Maruho (Vancouver)
Omiganan (MBI-226)	Synthetic cationic peptide derived from bovine indolicidin	catheter-related bloodstream infections	III (being repeated)	Microbiologix Biotech Vancouver, BC
Omiganan (CLS001)	Synthetic cationic peptide derived from bovine indolicidin	Severe acne and rosacea; anti-inflamotory	II/III	Cutanea Life Sciences/Migenix
MBI 594AN	Indolicidin (Bovine)	topical Rx for acne	IIB	Microbiologix Biotech Vancouver, BC, Canada
Iseganan (IB-367)	synthetic 17-mer peptide from pih protegrin 1	oral mucositis in patients undergoing radiation therapy	III	Ardea Biosciences
OP-145	Synthetic 24-mer peptide derived from LL-37 for binding to lipopolysaccharides or lipoteichoic acid	Chronic bacterial middle-ear infection	II (complete)	OctoPlus (Leiden, The Netherlands)
Novexatin	Cyclic cationic peptide, 1,093 daltons	Fungal infections of the toenail	I/II	NovaBiotics (Aberdeen, UK)
Lytixar (LTX-109)	Synthetic, membrane-degrading peptide	Nasally colonized MRSA	I/IIA	Lytix Biopharma (Oslo)
NVB302	Class B lantibiotic	*C. difficile*	I	Novacta (Welwyn Garden City, UK)
MU1140	Lantibiotic	Gram-positive bacteria (MRSA, *C. difficile*)	Preclinical	Oragenics (Tampa, Florida)
hlF1-11	Cationic peptide fragment comprising amino-terminal amino acids 1–11 of human lactoferricin	Bacteraemia and fungal infections in immuno-compromised haematopoetic stem cell transplant recipients	II (complete)	

Arenicin	21 amino acids; rich in arginine and hydrophobic amino acids	Multiresistant Gram-positive bacteria	Preclinical	Adenium Biotech Copenhagen
Avidocin and purocin	Modified R-type bacteriocins from *P. aeruginosa*	Narrow spectrum antibiotic for human health and food safety	Preclinical	AvidBiotics (S. San Francisco, California)
IMX924	Synthetic 5-amino-acid peptide innate defense regulator	Gram-negative and Gram-positive bacteria (improves survival and reduces tissue damage)	Preclinical	Iminex (Coquitlam, British Columbia, Canada)
IMX-942	Synthetic 5-amino-acid peptide derived from innate defense regulator IDR_1 and bactenecin	Nosocomial infection, febrile neutropenia and Immunomodulation during mucositis	II	Iminex (Coquitlam, British Columbia, Canada)
PAC-113	Synthetic 12-mer peptide derived from Histatins 3 and 5 (Human)	Mouth rinse for oral candidiasis in HIV patients	II (complete)	Pacgene Biopharmaceuticals (life science) Vancouver Canada
Xoma-629	derivative of Human Bactericidal Permeability-increasing protein (BPI)	Impetigo	II	Xoma (US) Berkeley, CA
Neuprex (rBPI21)	BPI (Human)	To reduce inflammatory complications in pediatric open heart-surgery patients.	I/II	Xoma (US) Berkeley, CA
PMX-30063	Arylamide oligomer mimetic of a defensin	Acute bacterial skin infections caused byStaphylococcus spp.	II	PolyMedix
Delmitide (RDP58	Semisynthetic D-amino acid decapeptide derived from HLA class I B2702	Inflammatory bowel disease	II (complete)	Genzyme
Ghrelin	Endogenous host-defense peptide	Airway inflamation, chronic respiratory infection and cystic fibrosis	II	University of Miyazaki, japan; Papworth Hospital Cambridge
Plectasin	Fungal defensin; candidate in development is an amino-acid substitiution variant	Bacterial diseases	Preclinical	Novozymes
HB1345	Synthetic lipohexapeptide	Acne; broad spectrum antibiotic	Preclinical	Helix Biomedix

1.9 Bacteriocins

Bacteriocins were among the first antimicrobial peptides to be isolated and characterized. Strains of *Escherichia coli* were observed to inhibit one another (Gratia 1925). A few years later, lactococci were reported to produce antibacterial substances (Rogers 1928). These compounds were initially called antibiotics. The term 'bacteriocin' was however introduced in the 1950s to differentiate these ribosomally synthesized peptides (Whitehead 1933) from classical antibiotics (Jacob et al. 1953). Bacteriocins have been defined as antibacterial proteins produced by some bacterial strains, which are bactericidal to other, often closely related bacterial strains, with different inhibitory mechanisms (Hoover and Steenson 1993).

Bacteriocins have been grouped into different classes. Class I bacteriocins are described as small membrane peptides with unusual amino acids (with sulfur containing ring, i.e. lanthionine rings). Class II bacteriocins are also small (20-60 amino acids), heat-stable, positively charged peptides but do not contain lanthionine amino acids. They are further divided into subclasses IIa, IIb, IIc, IId and IIe. Class III bacteriocins are large heat-labile proteins. Class III is divided into subclasses IIIa and IIIb. Class IV bacteriocins are complex bacteriocins containing lipid or carbohydrate moieties and class IV is a newly established group as they have not been adequately characterized at the biochemical level (Nes and Holo 2000, Sablon et al. 2000, Sonomoto and Zendo 2011).

Nisin is the most studied and best understood bacteriocin (Jack et al. 1995). The term nisin was coined to describe the *N* inhibitory substance of *L. lactis* (Mattick and Hirsch 1947). It is a Class I lantibiotic containing 34 amino acids with 5 unusual lanthionine rings and some dehydrated residues (Liu and Hansen 1990, Breukink et al. 1998). Nisin molecules facilitate the loss of intracellular solutes by assembling in the cell membrane

to form a barrel-like structure (Moll et al. 1999). Although many bacteriocins can only inhibit closely related strains of species, nisin is active against a variety of Gram-positive bacteria. However, nisin producing lactococci are resistant to its activity. This resistance is conferred by immunity proteins (Nis I, F, E and G) (Dodd et al. 1996, Saris et al. 1996).

1.10 Antimicrobial production in rumen bacteria

The first evidence of bacteriocin production among rumen isolates was found in *Strep. bovis* (Iverson and Millis 1976). More recently, a bacteriocin was purified from a bacterium originally thought to be *Strep. bovis* but is rather from an isolate closely related to *Strep. gallolyticus* LRC0255 (Whitford et al. 2001). The bacteriocin of LRC0255 (bovicin 255) is a positively charged molecule, which inhibits nisin-sensitive but not nisin-resistant *Strep. bovis* cells (Mantovani et al. 2001). Another bacteriocin, bovicin HC5, isolated from a strain closely related to *Strep. bovis* produced a bacteriocin-like substance that could inhibit both nisin-sensitive and nisin-resistant *Strep. bovis* strain JB1 (Mantovani et al. 2001, Mantovani et al. 2010). Bacteriocin production has also been reported in *Strep. bovis* isolates from Australian ruminants (Joachimsthal et al. 2010).Bovicin HC5 has also been shown to have activity against *Sal. Typhimurium* (Prudêncio et al. 2015).

Many strains of *Buty. fibrisolvens* have also been reported to produce bacteriocins that could inhibit other Butyrivibrios (Kalmokoff et al. 1996). Two Butyrivibriocins purified from *Buty. fibrisolvens OR79* (a lantibiotic) and *Buty. fibrisolvens AR10* (homologous to acidocin, a Class IIc bacteriocin produced by *L. acidophilus* respectively) have a wide spectra of activity and were able to inhibit a wide variety of Gram-positive ruminal bacteria (Kalmokoff et al. 1997, Kalmokoff and Teather 1997, Kalmokoff et al. 1999).

In another study, *Buty. fibrisolvens* strain Jl5 produced a bacteriocin that inhibited *Buty. fibrisolvens AR10* and also catalyzed potassium efflux and a decrease in ATP and electrical cell membrane potential in *Buty. fibrisolvens 49* (Rychlik and Russell 2002).

In other studies, a strain of *R. albus,* which produced a soluble, heat stable proteinaceous substance that inhibits the growth of *R. flavefaciens,* was discovered (Odenyo et al. 1994, Odenyo et al. 1994). *R. albus* strains have also been shown to have inhibitory effects against *Buty. fibrisolvens* (Chan and Dehority 1999). Bacteriocin producing *Ent. faecium* strains CCM4231 and BC25 have also been isolated from the rumen although *Ent. faecium* is not a dominant ruminal bacterium (Laukova and Czikkova 1998, Morovsky et al. 1998). These strains both inhibited *Strep. bovis* although the latter strain appeared to be bacteriostatic rather than bactericidal (Laukova and Czikkova 1998). Many lactobacilli, especially *L. fermentum* have been shown to inhibit the growth of *Strep. bovis* (de Klerk and Smit 1967, Wells et al. 1997).

It has been speculated that bacteriocins produced by ruminal bacteria may provide effective alternatives to antibiotics as feed supplements in ruminants (Teather and Forster 1998, Pieterse and Todorov 2010). In order for ruminal bacteriocins to be effective, they must be relatively stable, have a broad spectrum, and remain active (not subject to resistance) (Russell and Mantovani 2002). In this era of antimicrobial resistance and decreased drug discovery, one may also speculate that bacteriocin produced by rumen bacteria may provide alternatives to exploring novelty in drug discovery for human use.

1.11 Metagenomics for biotechnology

Several ruminal bacteriocins have been found to date, but only from bacteria that have been cultivated. In order to increase their discovery rate, and that of other novel

antimicrobials, culture independent methods can be used to assess the rumen microbiota. It is possible to access and explore the total genetic information of this unexplored, uncultured microbiota (i.e. the microbial community DNA), the so-called 'Metagenome' (Handelsman et al. 1998, Ekkers et al. 2012) through direct isolation and cloning of all micro-organisms living in a defined habitat without any need to isolate and cultivate any cells (Handelsman et al. 1998, Biddle et al. 2008).

Metagenomics, a term first used by Handelsman and colleagues (Handelsman et al. 1998), is a methodology that applies genome sequencing or assays of functional properties to culture-independent analysis of complex and diverse ('meta') populations of microbes (Wooley and Ye 2009). Metagenomic research bypasses the need for isolation and cultivation of microorganisms (Simon and Daniel 2011) and attempts to directly determine the whole collection of genes within an environmental sample (Wooley and Ye 2009). By enabling an analysis of many as yet unculturable and often unknown microbes, metagenomics is revolutionizing the field of microbiology (Handelsman 2004, Cowan et al. 2005, Wooley and Ye 2009, Suenaga 2012). It promises to provide new and diverse enzymes and biocatalysts as well as bioactive molecules and has the potential to make industrial biotechnology an economic, sustainable success (Li and Qin 2005, Lorenz and Eck 2005, Langer et al. 2006, Ferrer et al. 2009, Chistoserdova 2010, Singh 2010).

Metagenomic analyses are usually initiated by the isolation and purification of DNA from the environment (Streit and Schmitz 2004). The genetic material is then transferred into surrogate organisms to generate a metagenome clone library. The classical approach includes the construction of small insert libraries <10kb in a standard sequencing vector and in *E. coli* as a host strain (Henne et al. 1999, Handelsman 2004, Cowan et al. 2005, Daniel 2005, Hugenholtz and Tyson 2008). Additional hosts

including *Streptomyces lividans*, *P. putida* and *Thermus thermphilus*, amongst others have also been employed (Wang et al. 2000, Martinez et al. 2004, Albers et al. 2006, Angelov et al. 2009, Craig et al. 2010). Large insert libraries have been generated using cosmids (Entcheva et al. 2001, Courtois et al. 2003), fosmids (Quaiser et al. 2002, Quaiser et al. 2003, Treusch et al. 2004) (both up to 40kb) and bacterial artificial chromosomes (>40kb) (Rondon et al. 2000).

Screens of metagenomic libraries in search for biological products have been performed using two different approaches, i.e. using (i) sequence-based screening (mining of the genetic information by sequencing and PCR) and (ii) functional screening of clones (function-based screening) (Schloss and Handelsman 2003, Streit and Schmitz 2004, Daniel 2005, Schmeisser et al. 2007, Ferrer et al. 2009, Kakirde et al. 2010, Singh 2010, Suenaga 2012) (Figures 1.15 and 1.16). Significant progress has been made using both sequence- and function-based approaches, allowing the isolation of high quality DNA from a variety of environments including the rumen (Duan et al. 2009, Hess et al. 2011).

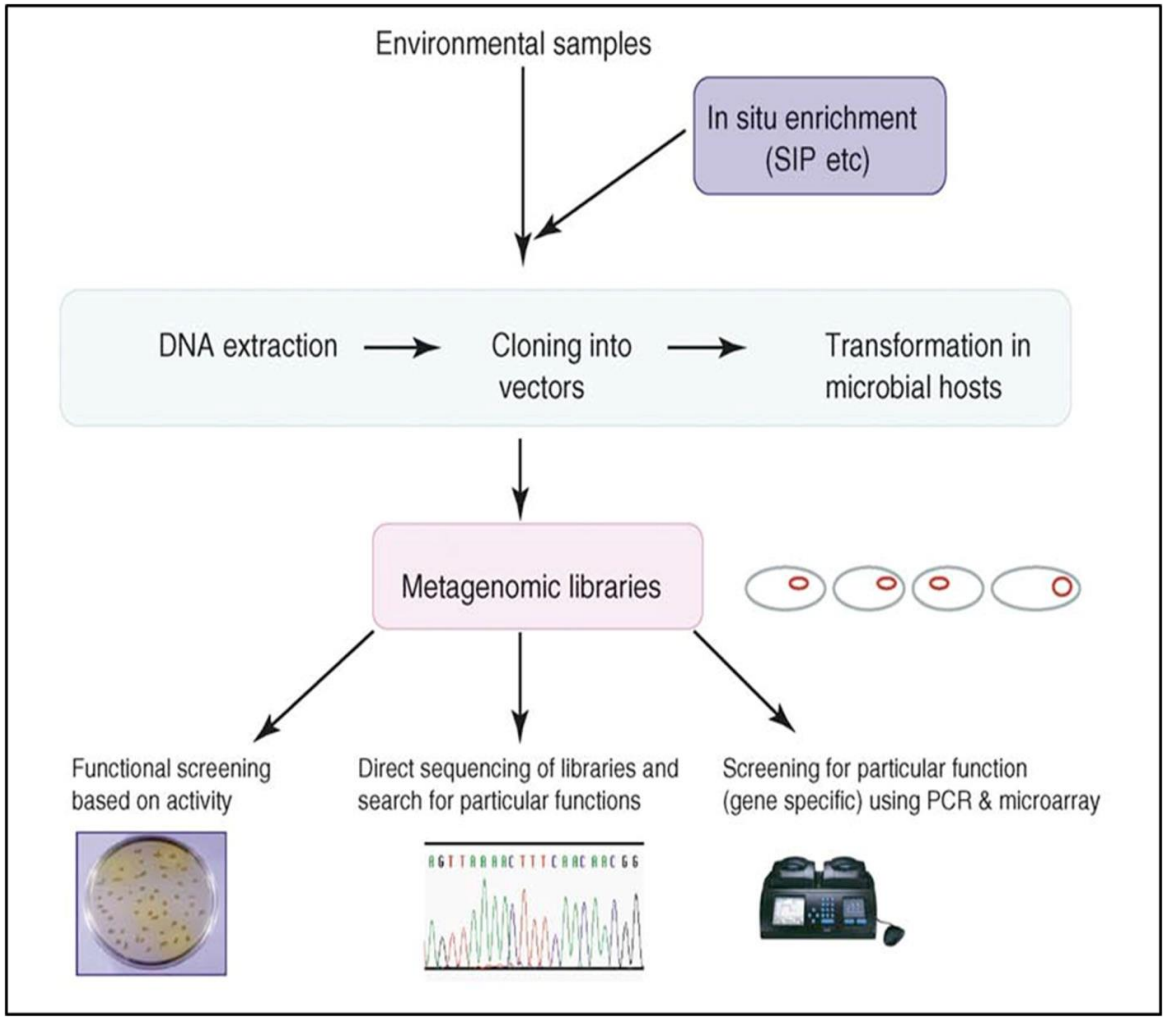


Figure 1.15 Metagenomic library construction and subsequent screening procedures. Source: (Singh 2010).

1.11.1 Sequence-based screening

In this approach, the search for a particular function or protein is performed by mining the metagenomic sequence data. Once putative homologues are found, the exact sequence information can be obtained by PCR amplification and expression in surrogate organisms (Singh 2010). This approach has been successfully used in the discovery of several enzymes, including chitinases, hydrogenases, lipases and carboxypeptidases (Lefevre et al. 2007, Ferrer et al. 2009, Maroti et al. 2009, Schmidt et al. 2010, Prive et al. 2015). However, its major drawback is that it depends on the availability of homologous sequence data. Therefore, it would fail to identify novel enzymes that have

the same function, but a different structure than known enzymes (Schmeisser et al. 2007, Singh 2010).

1.11.2 Function-based approach

Here, a function-based assay in which surrogate organisms are tested for a particular activity, such as reactions catalyzed by particular enzymes, or properties attributed to particular metabolites like antibiotics and anti-tumor agents is used (Singh 2010). The major problem of this approach is the logistics and facilities required to screen millions of clones for desired functions (Singh 2010). More so, function-based metagenomic studies often fall short of yielding products with sufficient novelty for biotechnological processes (Beloqui et al. 2008, Hill and Fenical 2010, Singh and Macdonald 2010). This is probably due to low level of gene expression in the library host (van Elsas et al. 2008) as genes may not be translated properly, particularly if they are not bacterial in origin or because the sensitivity of the screening methods used is too low for detecting gene expression (Gabor et al. 2004). However, as sequence information is not required, this approach alone bears the potential to identify entirely novel classes of genes encoding known or novel functions (Handelsman 2004, Riesenfeld et al. 2004, Daniel 2005, Ferrer et al. 2009, Gloux et al. 2011). Three different function-driven approaches (Simon and Daniel 2009), which have been used to recover novel biomolecules, include phenotypical detection of desired activity (mostly using chemical dyes) (Beloqui et al. 2010, Liaw et al. 2010, Gloux et al. 2011), heterologous complementation of host strains or mutants (Riesenfeld et al. 2004, Wang et al. 2006, Simon et al. 2009, Chen et al. 2010), and induced gene expression (Uchiyama et al. 2005, Williamson et al. 2005).

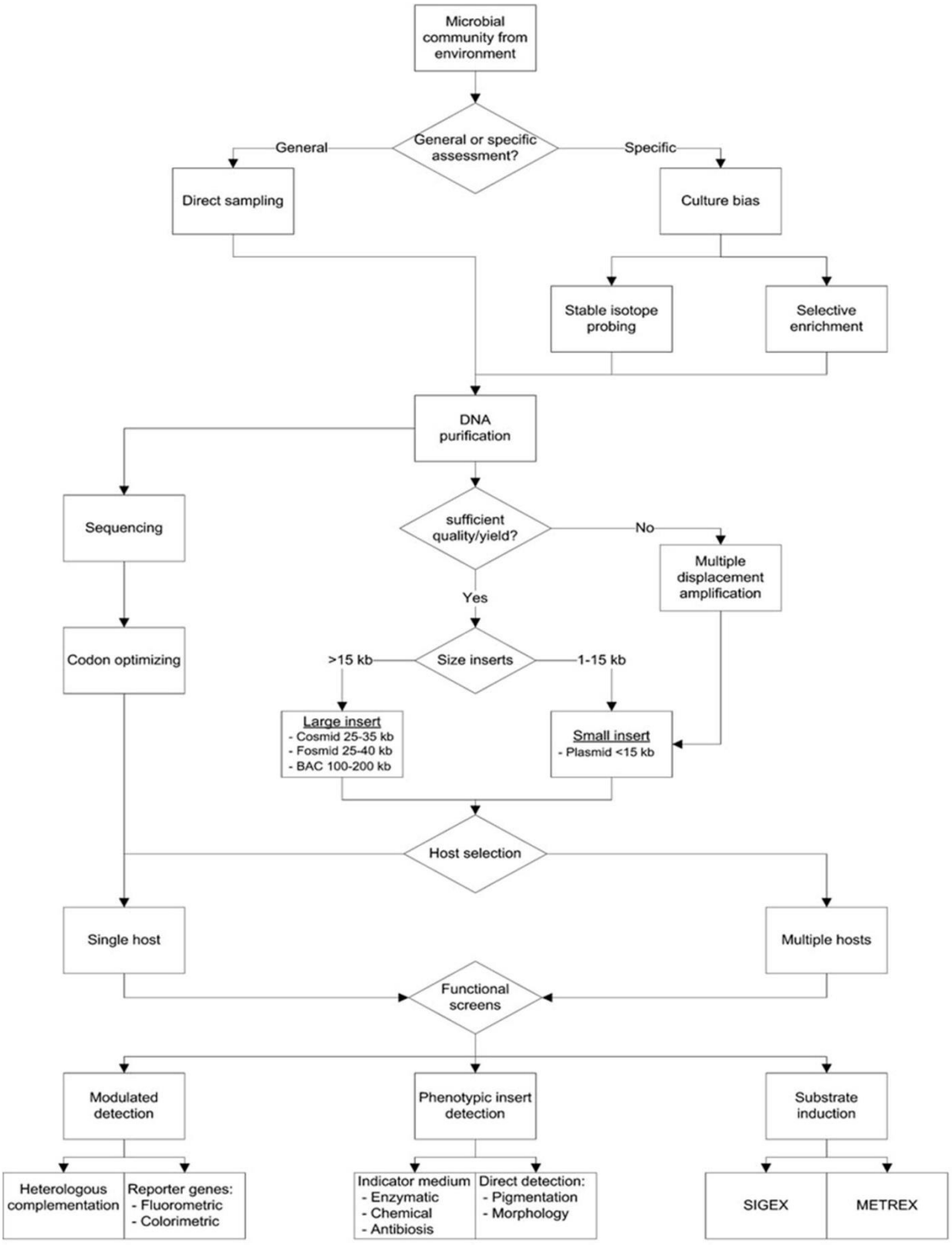


Figure 1.16 Schematic overview of the major function-based metagenomic assessment strategies. Source: (Ekkers et al. 2012).

1.12 Metagenomics- exploring the rumen bacteria: a potential source for novel antimicrobials

Direct genomic or metagenomic cloning also offers the opportunity to capture genes encoding the synthesis of novel antimicrobials (Schloss and Handelsman 2003), whether from species with already known antimicrobial properties (bacteriocin production), or from completely new species. Indeed turbomycin (Gillespie et al. 2002), long-chain N-acetylated amines (Newman and Cragg 2007), isonitrile functionalized antibiotics (Hugenholtz et al. 1998), and the pigments violacein and indigo (Rappe and Giovannoni 2003) were originally isolated from soil-based metagenomic libraries. The rumen is a rich microbiota of culturable and non-culturable bacteria, which behave cooperatively and competitively and so presents a potentially rich source of novel products, particularly antimicrobials. However, little has been done to prospect the rumen bacteria for novel antimicrobials and this project aims to address these areas in depth.

1.13 Aims of study

Due to the fact that bacterial resistance to antimicrobials poses an important threat to human health, there is a need to urgently develop tools with which we can develop novel antimicrobials and predict the potential for the evolution of resistance to novel drugs and treatment schemes (Levy and Marshall 2004, Martinez et al. 2007). We now have knowledge of the diverse ruminal microbial species, the interactions between them and their potential as possible sources of antimicrobial compounds. Modern methods (i.e. genomic approaches) therefore, can be used to explore novelty in the rumen, reduce the likelihood of resistance developing by choosing antibiotic targets for which the resistance mechanism confers a high fitness cost and for which the rate and extent of compensation is low (Andersson and Hughes 2010).

The aim of this thesis therefore, was to prospect rumen bacteria for novel antimicrobials, firstly from a fosmid-based rumen bacterial metagenomic library previously created in the Institute of Biological, Environmental and Rural Sciences (IBERS), and secondly from an already existing 268GB rumen metagenomic sequence dataset (Hess et al. 2011) using both functional and sequence based metagenomics and advanced bioinformatic mining tools respectively. The work also sought to characterize the antimicrobial activities and mode of action of the antimicrobial proteins identified. The information generated may provide novel antimicrobial compounds and will be relevant to the treatment of multidrug resistant bacterial infections; potentially overcoming resistance issues with predecessors.

CHAPTER TWO

General materials and methods

2.1 Bacterial strains

Bacterial strains used for antimicrobial activity testing in this project were all gifts from Bath University. They include Methicillin sensitive *Staphylococcus aureus* (MSSA) RN4220, *Escherichia coli* K12, *Salmonella enterica* serovar Typhimurium SL1344, *Enterococcus faecalis* JH2-2 and *Listeria monocytogenes* NCTC 11994 (serovar 4b). *E. coli* TOP10 (Invitrogen, Carlsbad CA, USA) was used for cloning (to host expression vectors for protein expression). Other bacterial strains used include Epidemic Methicillin resistant *Staphylococcus aureus* EMRSA-15 and *Pseudomonas aeruginosa* (15692) PAO1 strain H103. For the screening assays, a mini-*Tn5*-*lux* mutant in *P. aeruginosa* PAO1 strain H103 (*P. aeruginosa* PAO1 strain H1001) was used because of its high luminescent property (St. George's University, London, UK).

2.2 Antimicrobial agents and chemicals

Ampicillin, Chloramphenicol, Ciprofloxacin, Vancomycin, Polymyxin B and Tobramycin were purchased from Sigma-Aldrich (Poole, Dorset, United Kingdom). Bactenecin Bac2A was purchased from Eurogentec Ltd Southampton UK). All stock solutions were dissolved in the appropriate solvent prior to dilution in sterile distilled water (Andrews, 2001). Table 2.1 lists all the antimicrobial agents used in the study and their solvents.

Table 2.1 Antimicrobial agents used in this study

Antimicrobial agent	Solvent
Vancomycin	H_2O
Tobramycin	H_2O
Polymyxin B	H_2O
Ampicillin	H_2O
Chloramphenicol	H_2O
Bactenecin Bac2A	H_2O
Ciprocloxacin	H_2O

2.3 Bacteriological media and culture conditions

Mueller Hinton (MH) (Sigma-Aldrich UK) and Luria Bertani (LB) broth and agar (Fisher Scientific Leicestershire, UK) were routinely used for all bacterial cultures. When leakage assays were performed under buffered conditions, 5mM HEPES (pH 7.2) supplemented with 5mM glucose was used (Wu and Hancock 1999). Media were prepared and sterilized according to the manufacturers' instructions.

Bacterial strains were grown using standard conditions unless otherwise specified. Broth cultures were incubated at 37^{o}C for 18-20 hours with aeration and cultures on solid media were incubated at 37^{o}C for 18-24 hours, except for *List. monocytogenes* which was incubated at 30^{o}C.

2.4 Rumen metagenomic library construction

The fosmid metagenomic library used in this study has been previously constructed at the Institute of Biological Environmental and Rural Science (IBERS) at Aberystwyth University (Privé 2012, Privé et al. 2015). Metagenomic libraries were constructed from DNA extracted from bovine rumen content fractionated into strained rumen fluid (SRF),

solid-attached bacteria (SAB) and liquid associated bacteria (LAB), ligated into a fosmid vector and subsequently transformed into an *E. coli* host (Prive et al. 2015).

Briefly, rumen contents were collected from four rumen-fistulated, non-lactating Holstein cows (average weight of 731 kg) housed at Trawscoed experimental farm (Aberystwyth, Ceredigion, Wales). The animals were fed a diet composed of a mixture of grass silage and straw (75:25) *ad libitum* and ~1 kg of sugar beet nuts was fed at 0700 h daily with constant access to fresh water. Sampling was completed 2 h after concentrate feeding. SRF, SAB and LAB were harvested as described by (Huws et al. 2010).

Metagenomic DNA was extracted from 200 µl of SRF, SAB and LAB using the BIO101 FastDNA® Spin Kit for Soil (Qbiogene, Cambridge, UK) following the supplier's protocol, except that after the first step, the samples were shaken three times for 30s, in a FastPrep bead beater (Qbiogene, Cambridge, UK) at 6 m/s with cooling on ice for 30s between each shake. The libraries were constructed using the CopyControl™ pCC1FOS™ vector and the reagents supplied in the CopyControl™ Fosmid Library Production Kit (Epicentre, Cambio Ltd., Cambridge, UK), following the supplier's recommendations. All clones were picked using a colony picker Genetix QPix2 XT (Genetix Ltd., New Milton, England), and subcultured for 20 h in 384-well plates (Genetix Ltd., New Milton, England) containing LB broth with 12.5 µg/ml chloramphenicol and 20% glycerol. They were then stored at -80°C.

Fosmid libraries consisted of 7,744, 8,448 and 7,680 clones with an average insert size of 30 to 35 kbp for SRF, SAB and LAB respectively (Prive 2011). Only the solid attached bacteria (SAB) fosmid library with 8,448 clones was used in this study.

2.5 Bacteriological techniques

2.5.1 Determination of susceptibilities to antimicrobial agents

Minimal inhibitory concentrations (MICs) were determined using two-fold serial dilutions of antimicrobial agents in LB broth as described by the National Committee of Clinical Laboratory Standards (NCCLS) (NCCLS, 1997). MICs for antimicrobial peptides and comparator antimicrobials were measured using a modified broth microdilution method in LB broth. Briefly, the peptides were dissolved and stored in sterile microcentrifuge tubes. The assay was performed in sterile U bottom polypropylene 96 well microplates with lids (Greiner Bio One Ltd, Stonehouse, UK). Serial dilutions of antimicrobials were performed in sterile distilled water at 10-fold the desired final concentrations. Ten microlitres of this 10 fold dilutions were added to each well of the 96 well microplate containing 90 µl of LB broth per well. Bacteria from overnight culture (adjusted to 1×10^8 CFU/ml) were added to the plate at a final inoculum concentration of 5×10^5 CFU/ml (Cherkasov et al. 2008, Wiegand et al. 2008). The MIC was defined as the lowest concentration of drug that inhibited visible growth of the organism after an 18-24 hour incubation at appropriate temperature.

2.6 Molecular biology materials and methods

2.6.1 Vectors

The pTrcHis TOPO® vector (Invitrogen, Carlsbad, CA, USA) was used to clone PCR products for protein expression as described in section 4.2.2.1.

2.6.2 DNA and plasmid DNA extraction

DNA was extracted using a QIAprep® Spin Miniprep kit (Qiagen, Crawley, UK). Bacterial overnight cultures were pelleted by centrifugation at 4,000 x g for 10 minutes. The supernatant was discarded; pelleted cells were resuspended in 250 µl of Buffer P1

and transferred to a sterile 1.5ml microcentrifuge tube. Buffer P2 (250 µl) was then added and thoroughly mixed by inverting the tube and allowed to stand for 5 minutes. This was followed by the addition of 350 µl Buffer N3 to the tube and mixed thoroughly to neutralize the lysis reaction. The tube was then centrifuged in a bench-top microcentrifuge (Heraeus Biofuge Fresco microlitre centrifuge, Thermo Scientific UK) at 13,000 x g for 10 minutes. The supernatant from the previous step was applied to a QIAprep column and centrifuged at 13,000 x g for 1 minute to bind the plasmid DNA to the column. Buffer PB (500μl) was added to the column and centrifuged at 13,000 x g for 1 minute. The flow-through was discarded and 750 µl of buffer PE was added to the column and centrifuged at 13,000 x g for 1 minute to wash the bound DNA. Centrifugation step was repeated after discarding flow through to remove residual wash buffer. The QIAprep column was placed in a sterile 1.5ml microcentrifuge tube and 50 µl of elution buffer EB was added to the centre of the column to elute the plasmid DNA. This was allowed to stand for 1 minute at room temperature and then centrifuged at 13,000 x g for 1 minute. The flow through (extracted DNA) was stored at -20°C for further analysis. As advised by the supplier, the elution Buffer EB was pre-warmed at 70°C before use for longer plasmid DNA (up to 30-35 kb) extraction.

2.6.2.1 DNA Extraction from milled rumen samples

When DNA was to be extracted from milled rumen samples, the FastDNA® SPIN Kit for Soil (MP Biomedicals) was used to extract DNA as described by the manufacturer. Briefly, up to 100 mg of rumen sample was added to a Lysing Matrix E tube. Sodium Phosphate Buffer (978 µl) and MT Buffer (122 µl) were then added to the tube and homogenized in a FastPrep® Instrument for 40 seconds at a speed setting of 6.0. The tube was then centrifuged at 14,000 x g for 5-10 minutes to pellet debris. The supernatant was transferred to a clean 2.0 ml microcentrifuge tube, to which 250 µl

Protein Precipitation Solution (PPS) was added and mixed by inverting the tube by hand 10 times. This was followed by centrifugation at 14,000 x g for 5 minutes to pellet precipitate. The supernatant was then transferred to a clean 15 ml tube for better mixing and DNA binding. Resuspended Binding Matrix suspension (1.0 ml) was then added to the supernatant in the 15 ml tube and inverted by hand for 2 minutes to allow binding of DNA after which it was allowed to stand in a rack for 3 minutes to allow settling of silica matrix. Some of the supernatant (500 µl) was then removed to waste before re-suspending Binding Matrix in the remaining amount of supernatant. The mixture was subsequently transferred (600 µl at a time) to a SPIN™ Filter and centrifuged at 14,000 x g for 1 minute. After centrifugation, the catch tube was emptied, and the pellet was carefully resuspended in 500 µl prepared SEWS-M (ethanol added) using the force of the liquid from the pipette tip and centrifuged again at 14,000 x g for 1 minute. The residual wash solution on the matrix was 'dried' by centrifugation at 14,000 x g for 2 minutes. The catch tube was then discarded and replaced with a new, clean catch tube and the SPIN™ Filter was air dried for 5 minutes at room temperature. Finally, 50 µl of DNase/ Pyrogen-Free Water (DES) was added to the SPIN filter and incubated for 5 minutes at 55˚C in a heat block or water bath (to increase DNA yield). This was followed by centrifugation at 14,000 x g for 1 minute to bring eluted DNA into the clean catch tube. Eluted DNA was stored at -20°C for extended periods or 4°C until use.

2.6.3 DNA quantification

DNA quantification after extractions and polymerase chain reaction (PCR) amplifications were carried out using BioTek's Epoch™ Multi-Volume Spectrophotometer System run by Gen5™ 2.0 Microplate Data Analysis software (BioTek Instruments, Inc. Vermont, USA). DNA samples (2 µl) were placed on the Take3™ Multi-Volume Plate and absorbance recorded at 260 280 and 320 nm. The

ratio 260/280 nm (to evaluate purity), absorbance values, and concentrations of samples were calculated by the Gen5™ software.

2.6.4 Agarose gel electrophoresis

The size and integrity of extracted DNA and PCR products were visualised and assessed by agarose gel electrophoresis using a 1.5% (w/v) agarose in TAE buffer (40 mM Tris, pH 8.0, 20 mM acetic acid, 1 mM EDTA, BioRad Ltd., Hemel Hempstead, UK). The agarose solution was dissolved in a microwave on high heat until clear (~ 3 minutes) and cooled to approximately 50°C, after which GelRed™ Nucleic Acid Gel Stain 10,000X in water (Biotium Inc. USA) was added and mixed thoroughly to a final concentration of 1X GelRed™ (1:10000 dilution). The appropriate sized comb was then placed into a casting tray containing the agarose solution and allowed to polymerise at room temperature for ~ 30 minutes depending on gel size. When solidified, the combs were removed and the gel was transferred to an electrophoresis tank (Horizontal Electrophoresis Unit, Sigma-Aldrich) which contained 1X TAE above the level of the gel.

Samples for agarose gel electrophoresis were prepared by mixing a 4:1 ratio (v/v) with blue/orange 6X loading dye (Promega UK Ltd., Southampton, UK), containing 0.4% orange G, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 15% Ficoll® 400, 10 mM Tris-HCl (pH 7.5) and 50mM EDTA (pH 8.0). This step was not required for PCR products prepared using pre-stained Taq polymerase or PCR Mastermix (e.g. MyTaq™ Red Mix). The reference ladder (1kb ladders, Promega UK Ltd., Southampton, UK) was mixed in a 4:1 ratio (v/v) with the same loading dye. A total of 5 µl of samples and ladder were loaded into individual wells of the agarose gel.

Ladder and samples were loaded on the gel, and electrophoresis was performed at a constant voltage (100V) until the dye front was near the end of the gel. Gel image was taken after exposure to UV using the Gel Doc^{TM} XR^{+} system (BIO-RAD Hertfordshire, UK) and analysed by Quantity One 1-D Analysis Software (BioRad Ltd., Hemel Hempstead, UK).

2.6.5 DNA gel extraction and purification

DNA Gel Extraction and purification was carried out using the QIAquick Gel Extraction Kit (Qiagen). DNA fragments were excised from the agarose gel with a clean sterile, sharp scalpel. The gel slice was weighed and placed in a colourless microcentrifuge tube. Three volumes of Buffer QG was added to 1 volume of gel (100 mg ~ 100 µl). For example, add 300 µl of Buffer QG to each 100 mg of gel (the maximum amount of gel slice per QIAquick column being 400 mg). The gel slice was then incubated at 50°C for 10 minutes or until it was completely dissolved (by vortexing the tube every 2–3 minutes during the incubation). Attention was paid to the yellow coloration of the mixture which indicates that Buffer QG is at pH ≤7.5 the pH at which adsorption of DNA to the QIAquick membrane is most efficient. Isopropanol (1 gel volume) was added to and mixed with gel sample. For example, if the agarose gel slice is 100 mg, add 100 µl isopropanol. The sample was then applied to a QIAquick spin column in a 2ml collection tube and centrifuged at 13000 rpm for 1 minute. The flow-through was discarded and 0.5 ml of Buffer QG was again added to QIAquick column and centrifuged for 1 minute to remove all traces of agarose. The sample was washed by adding 0.75 ml of Buffer PE to the column and centrifuged for 1 minute after a 5 minute incubation time. The flow-through was once again discarded and the column centrifuged for an additional 1 minute at ≥10,000 x g (~ 13,000 rpm). Finally to elute the DNA, the QIAquick column was transferred into a clean 1.5 ml microcentrifuge

tube, and 30µl of Buffer EB (10 mM Tris·Cl, pH 8.5) was added to the centre of the QIAquick membrane, allowed to stand for 1 minute at room temperature and centrifuged for 1 minute at maximum speed and store at -20°C until required.

2.7 Synthesis of antimicrobial peptides/peptide library

In order to support the development of a class of drugs such as antimicrobial peptides, which can be used to discover new lead drug structures against multiresistant bacteria, a fast, easy and inexpensive method to synthesize peptides such as Spot-synthesis technology (Frank 1992, Kramer and Schneider-Mergener 1998) is necessary. The SPOT method follows standard fluorenyl-methoxy-carbonyl (Fmoc) chemistry on conventional cellulose sheets, allows the parallel synthesis of large numbers of addressable peptides in small amounts, can utilize more than 600 different building blocks and the cost per peptide is less than 1% of peptides synthesized conventionally on resin (Hilpert et al. 2007). Synthesis of unpurified peptides in the peptide library was completed at St. Georges University of London in collaboration with Dr Kai Hilpert's group and is described below. Synthesis of purified peptides on resin was done at >95% purity by GenScript USA Inc (Piscataway, New Jersey, USA).

2.7.1 Spot cellulose membrane activation

Using the spot synthesis technique allows several hundred peptides to be constructed onto cellulose membranes. However, to improve yields of peptides, the membranes first need to undergo a functionalization step in which an ester bond is formed between the hydroxyl groups found on cellulose membranes and the carboxyl group of the amino acid glycine.

Briefly, four, 10 cm by 15 cm membranes were cut from Whatman 50 ash-free paper and placed in a chemical resistant box. 2 x 2.97 g Fmoc-glycine was weighed into 2 x

50 ml Falcon tubes, into each of these a solution containing 45ml amine free dimethlyformamide (DMF), 1870 µl diisoproplycabomide (DIC) and 1586 µl N-methylimidiazole (NMI) was added, this solution was then topped up with enough DMF to achieve a total volume of 50 ml per Falcon. Twenty-five millilitres of the solution was added to each of the chemical resistant boxes containing the membranes, care was taken to remove any air bubbles that formed beneath the membrane. The boxes were placed on a platform rocker and left to gently rock overnight at room temperature.

The next day the solution was discarded from the boxes and membranes were washed three times with 20 ml DMF per box per wash. Next, 20 ml of 20% piperidine in DMF was added to each box and left to react for 20 minutes whilst undergoing gentle rocking at room temperature. After 20 minutes the solution was discarded and the membranes were subjected to five DMF washes and five ethanol washes (20ml per box per wash), all washes were performed on a rocking platform. Finally 20 ml 0.1% bromophenol blue in ethanol was added to each box. A blue staining of the membrane occurs in the presence of free amine groups of the glycine. Each membrane was then washed three times with 20 ml ethanol and left in the chemical resistant box to dry overnight in the fume hood.

2.7.2 Synthesis of peptides

Peptides were synthesised using solid phase Fmoc peptide chemistry using Intavis multipep RSi automated peptide synthesiser. All amino acids used were L-amino acids with acid labile side group protection and a base labile Fmoc amine protection. Peptides were synthesised either onto cellulose or resin supports according to the protocol stated in the “multipep RSi Manual” supplied by Intavis Bioanalytical instruments. The protocol for spot synthesis uses 0.5 M amino acid in N-methyl-2-pyrrolidone (NMP) which is activated by the robot using 1.1 M hydroxybenzotriazole (HOBt) in NMP and

1.1 M diisopropylcarbomide (DIC) in NMP. Deprotection of the amino Fmoc group happens prior to coupling and is carried out using 20% Piperidine in DMF. To improve peptide yields each amino acid coupling is carried out in duplicate. Uncoupled amino acids were acetylated at the end of each dual coupling cycle with 5% acetic anhydride in DMF. This process takes place in a cyclic manner until the peptide synthesis is complete, a schematic representation can be seen in Figure 2.1.

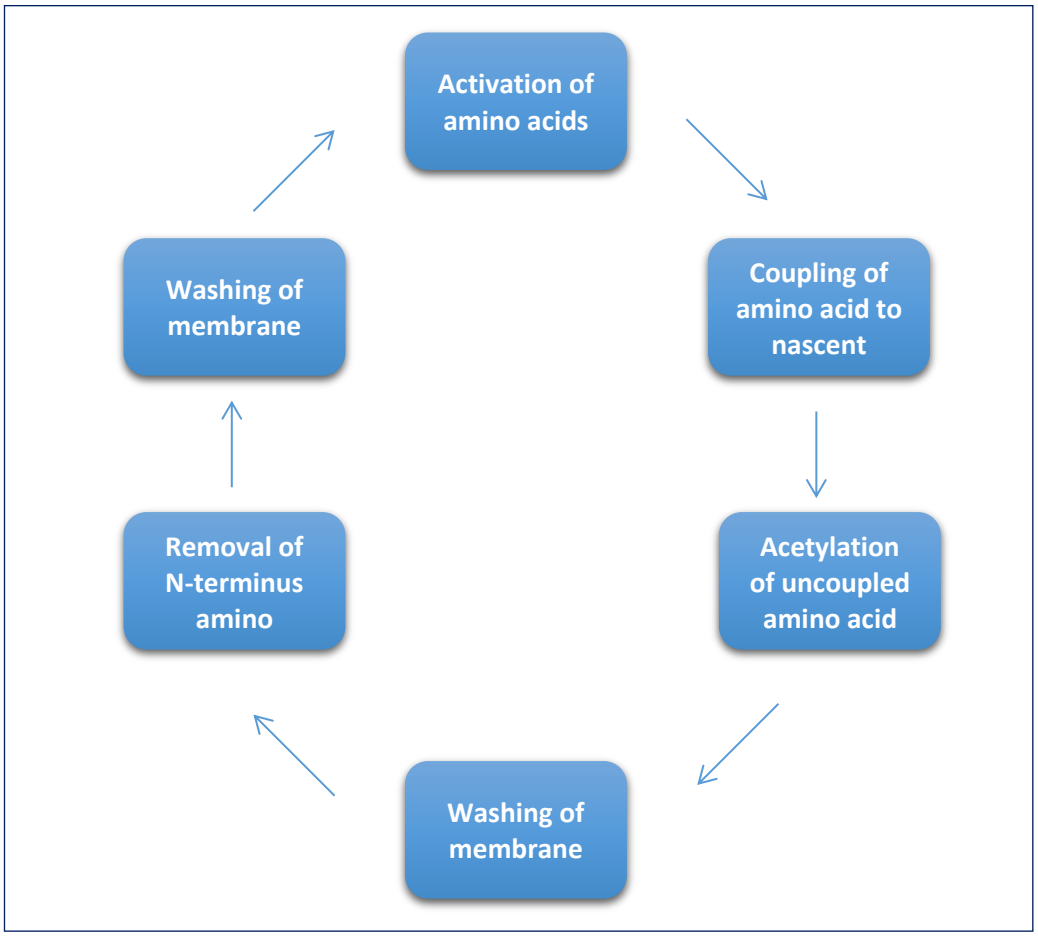


Figure 2.1 A schematic representation of the repeated cycle implemented in spot peptide synthesis.

During the first cycle, after Fmoc deprotection with piperidine and the completion of DMF washes, 1% bromophenol blue in ethanol was washed over the membranes to

detect free amino groups. These free amino groups indicate the position of the peptide spots and therefore were marked with a pencil so each peptide spot could be visualised and later punched out from the membrane.

The process of peptide synthesised on resin follows a similar protocol to the one described above, however, the chemicals used in the activation of the amino acids were O-(1H-Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) and N-methylmorpholine (NMM), no acetylation step was involved and all peptides were synthesised on 2-Cl-(Trt)-Cl resin using a 5 µM synthesis scale.

2.7.3 Side chain deprotection of cellulose bound peptides

Amino acids used during the peptide synthesis contain acid labile side chain protection moieties that prevent unwanted cross reactions and these were removed following the completion of the synthesis as follows: Under a fume hood cleavage solution A (5 ml Dichloromethane (DCM), 2 ml H_2O, 3 ml Triisopropyl silane (TIPS), 90 ml Trifluoroacetic acid (TFA)) was added to chemical resistant boxes containing the membranes (25 ml per box). The reaction was left for 30 minutes at room temperature and then the solution was removed. Cleavage solution B (45 ml DCM, 2 ml H_2O, 3 ml TIPS, 50 ml TFA) was then added to the boxes (25 ml per box). TFA is a highly corrosive acid and therefore special safety measures were employed during its use. After 2 hours, the solution was discarded and the membranes were washed three times in 20 ml DCM, followed by three washes in 20 ml DMF, then three washes in 20 ml ethanol and three washes in 20ml 10 mM Tris buffer (pH 7.5). The membranes were then left in sterile demineralised water overnight and air dried the following day.

2.7.4 Peptide cleavage from the membrane

The dried membranes were placed in a glass desiccator and a vacuum was created. Under a fume hood ammonia gas was gradually entered into a chemical resistant balloon using a regulator attached to the ammonia canister, then from the balloon the ammonia was transferred into the desiccator. This process was repeated until the pressure in the balloon and the desiccator reached equilibrium, i.e. the balloon did not fully deflate when attached to the desiccator, this indicates that the desiccator was saturated with ammonia. The membranes were left in the desiccator overnight and the next morning the desiccator was gradually opened over a period of 1 hour and then the membranes removed.

The individual spots (marked in pencil during the synthesis) were then punched out of the membrane using a single spot hole puncher, each spot was then individually transferred to a well of a polypropylene 96 well plate where it was solubilised in 200 µl sterile water. The final yield of peptide was determined by Thermo Scientific Nanodrop spectrophotometer according to the manufacturers protocol (ThermoFisher Scientific 2010). The yields for each peptide were approximately 300 µg/ml per spot, which equated to around 60 µg of crude (un-purified) peptide per spot.

2.7.5 Side chain deprotection and precipitation of peptides produced on resin

The deprotection and precipitation of peptides synthesised on resin was carried out according to the 24 column resin protocol from (Biosciences 2001). Briefly, the resin/peptide in columns were washed several times with *tert*-Butyl Methyl ether in the peptide synthesiser to remove all traces of DMF, the column module was then removed and dried overnight in a desiccator. The next day resin/peptides from each column were removed and placed into 15 ml falcon tubes. Afterwards, 12 ml of cleavage solution C

(11.1 ml TFA, 600 µl TIPS, 300 µl water) was prepared in a glass test tube, and into each Falcon tube containing peptide and resin 500 µl of cleavage solution C was added and left for 5 hours. After 5 hours had elapsed, the solution contained within the Falcon tubes was diluted with ice cold *tert*-Butyl Methyl ether to precipitate the peptide. The Falcon tubes containing the solution were then centrifuged for 5 minutes at 4000 rpm to form a peptide and resin pellet that was washed several times with ether. The peptide-resin pellet was then dried in a vacuum desiccator overnight and dissolved in H_2O the following day (at which point the resin could be removed by filtration). The peptides in solution were frozen in a -80^0C freezer and lyophilised in an Edwards Freeze Dryer Modulo the following day. The peptide powders were then ready to be weighed and solubilised in sterile demineralised H_2O at a stock concentration of 2 mg/ml. The peptide solutions were then stored in a -20^0C freezer until required. All work was carried out under a fume hood.

To determine the purity of the sample reverse phase analytical High performance liquid chromatography (HPLC) was carried out on the peptides. This was done using a C18 column (25 cm x 4.6 mm x 5 µm) with a 1 ml/minute flow rate and a 30 minute run (0 minute 0.1% TFA, 0 – 20 minutes 0.1% TFA – 80% acetonitrile, 20 – 22 minutes 80 – 100 % acetonitrile , 22 – 30 minutes 100 % - 0% acetonitrile).

2.8 Software used

BioEdit: BioEdit (version 7.0.5.3) is a sequence alignment editor for sequence analysis and editing, available at [http://www.mbio.ncsu.edu/bioedit/bioedit.html] (November 2010) (Hall, 1999).

BLAST: The Basic Local Alignment Tool Search (**BLAST**) finds regions of local similarity between sequences. The program compares nucleotide or protein sequences to

sequence databases and calculates the statistical significance of matches (Altschul *et al.*, 1997). It is available at [http://blast.ncbi.nlm.nih.gov/Blast.cgi].

CLUSTALW: The ClustalW2 program was used for multiple sequence alignments of DNA and protein sequences. It is available at [http://www.ebi.ac.uk/Tools/msa/clustalw2/].

RAST/MG-RAST: The Rapid Annotation using Subsystem Technology (**RAST**) is an automated service for annotating bacterial and archaeal genomes (Aziz *et al.*, 2008). The software identifies protein-encoding, rRNA and tRNA genes, assigns functions to the genes, predicts which subsystems are represented in the genome and uses this information to reconstruct the metabolic network of the organism. The service is available at [http://rast.nmpdr.org/] (November 2010).

SignalP 4.1 Server: predicts the presence of signal peptide cleavage sites in amino acid sequences from different organisms available at http://www.cbs.dtu.dk/services/SignalP/ (Petersen et al. 2011).

VecScreen: is a system for quickly identifying segments of a nucleic acid sequence that may be of vector origin. It is available at http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen. html.

MATLAB toolbox Gait-CAD (Mikut et al. 2008): including the Peptides Extension Package (Mikut 2010) were used for most of the computational analysis. Gait-CAD is free software and can be downloaded from http://sourceforge.net/projects/gait-cad/ but requires the commercial MATLAB software to work.

PHYRE2 and **PEP-FOLD**: were used for protein 3D structure prediction (Kelley and Sternberg 2009, Maupetit et al. 2009, Thevenet et al. 2012, Kelley et al. 2015)

PyMoL: was used for viewing protein structures (Schrödinger 2010).

2.9 Filtering steps followed in the identification of putative novel antimicrobials

Many methods and filtering steps and criteria were used in the identification of putative novel antimicrobilas in this study. These steps are discussed in the 'materials and methods' sections of Chapters three, four and five below. For ease of comprehension, Figure 2.2 below is a schematic flow chart highlighting the filtering steps followed in the study.

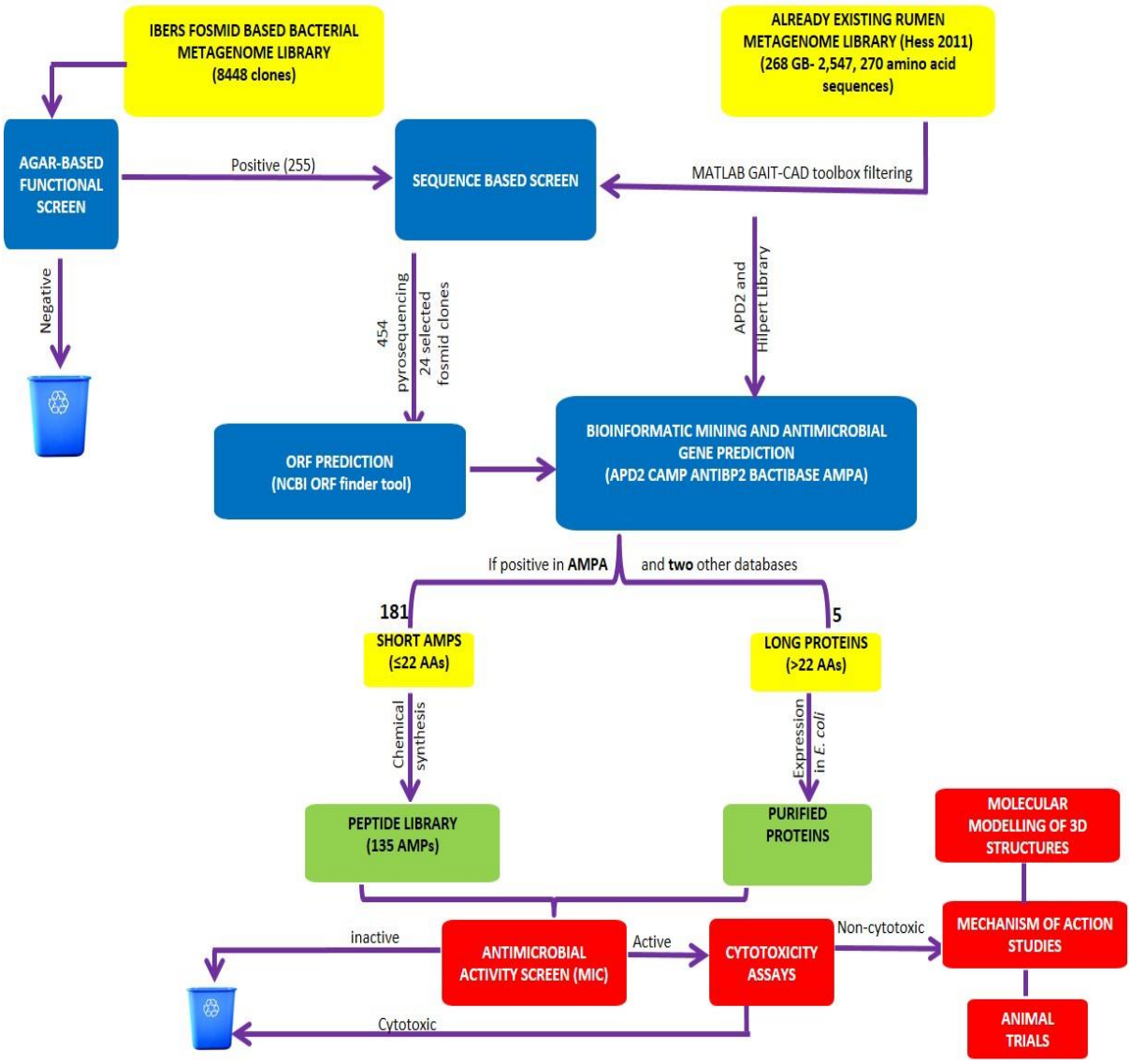


Figure 2.2 A schematic flow chat of filtering steps followed in the identification and characterisation of putative antimicrobials.

CHAPTER THREE

Discovery of novel antimicrobials from the rumen microbiome

3.1 Introduction and aim of chapter

The rumen is one of the most diverse ecosystems in nature, harboring a complex microbial community, composed of an immense diversity of bacteria, protozoa, fungi, and viruses (Church 1993, Sirohi et al. 2012). The diverse microbial community adapts to a wide array of dietary feedstuffs and management strategies, thus enzymes isolated from this ecosystem have the potential to possess very unique biochemical properties. Therefore the variation of microorganism communities in the rumen of cattle (*Bos taurus*) is of great interest because of possible links to economically or environmentally important traits (Ross et al. 2012).

Use of culture dependent methods to evaluate bacterial populations in the rumen substantially underestimates the diversity of microorganisms within the rumen as only around 10-20% of the species present are culturable at present (Edwards et al. 2004, Fernando et al. 2010, Sirohi et al. 2012). Direct sequencing of DNA, RNA and proteins extracted from an environmental sample through metagenomics and metaproteomics can provide snapshots of the complete microbiome and subsequently insights into changes of metabolic processes. Ever advancing nucleic acid-based technology and high-throughput methods have redefined our understanding and ability to describe various microbiomes, including the rumen microbiome. This has created new opportunities to investigate the complex relationships and niches within the microbial communities (McCann et al. 2014). It is important to improve our understanding of the functional capacity of the rumen microbiota, particularly of as yet unculturable bacteria, to fully understand the rumen microbiome. Consequently, genome sequencing of

microbes in the rumen is currently being undertaken by Hungate 1000 (www.hungate1000.org.nz/) and FibRumBa database (www.jcvi.org/rumenomics/) (Creevey et al. 2014). Increased sequencing efforts supported by continued platform development with longer reads at lower costs allow more widespread use of next generation sequencing technologies with corresponding proliferation of rates of data generation. Consequently, there has been a high demand and subsequent increase in the development of additional bioinformatics tools to process data and obtain meaningful functional results (McCann et al. 2014).

As with other ecosystems, several rumen metagenomic datasets have been generated in the last few years (Table 3.1). These metagenomic studies focus on nutrition, metabolism and the metabolic capabilities within the rumen microbiome due to the fact that this is pivotal to improving animal production efficiency and industrial fermentation. Unlike the soil and marine ecosystems metagenomes, which are heavily prospected for novel antimicrobial compounds (Gillespie et al. 2002, Barone et al. 2014, Ling et al. 2015), there are currently no rumen metagenomic studies investigating rumen antimicrobial genes and their potential as alternative treatments for multidrug resistant bacteria in humans and/or livestock.

Whole ecosystem metagenomics (marine and terrestrial alike) has shown great promise in the discovery of novel and biotechnologically important compounds including antimicrobial compounds. The rumen microbiome has also been cited as a resource of novel enzymes for cellulosic biofuel production (Rubin 2008, Hess et al. 2011, Del Pozo et al. 2012). However, rumen microbial diversity studies using 16S rRNA have mainly been focused on targeting genes responsible for biomass degradation, methane production and ruminant function.

Although antimicrobial activity (bacteriocins) have been identified among rumen bacteria (Iverson and Millis 1976, Odenyo et al. 1994, Kalmokoff et al. 1996, Kalmokoff et al. 1997, Wells et al. 1997, Kalmokoff et al. 1999, Whitford et al. 2001, Mantovani et al. 2002, Russell and Mantovani 2002, Rychlik and Russell 2002, Joachimsthal et al. 2010) using traditional methods, again the focus of these studies were mainly on the role of these bacteriocin production in fibre degradation, their effect on other ruminal bacteria and ammonia production, with only little research on their activity against human and livestock pathogens (Wang et al. 2011, Prudêncio et al. 2015). Exploration of these antimicrobial genes for their potential use in the treatment of human and livestock infections is therefore necessary. Given the advancement in genomic and sequencing technologies, and the increase in data generation, it is now possible to identify and investigate the antimicrobial properties of potentially novel antimicrobial compounds from the rumen and their applicability in the treatment of resistant bacterial infections.

The aim of this Chapter was to identify potential antimicrobial genes from the 8,448 clone fosmid based metagenomic clone library generated from cow rumen solid attached bacteria (SAB) at IBERS Aberystwyth University (Privé 2012, Prive et al. 2015) by a combination of agar-based functional screen, and sequence analysis. The chapter will also discuss bioinformatic mining and prediction of antimicrobial genes/proteins and antimicrobial peptides (AMPs) using various online databases and prediction tools first, in this fosmid library and secondly in the publicly available cow rumen metagenomic dataset from (Hess et al. 2011).

Table 3.1 Summary of recent bovine rumen metagenomic studies Adapted and modified from McCann et al. (2014)

Title	Year	Sequencing platform	Major finding	Reference
Gene-centric metagenomics of the fiber-adherent bovine rumen microbiome reveals forage specific glycoside hydrolases	2009	454 GS20	Significant differences exist in rumen metabolic potential between animals on the same diet. Many unique glycoside hydrolases are present in the rumen microbiome.	(Brulc et al. 2009)
Rumen bacterial diversity associated with changing from bermudagrass hay to grazed winter wheat diets	2010	454 GS FLX	Switching from hay to wheat diet affects bacterial composition and diversity structure in the rumen microbiome.	(Pitta et al. 2010)
Rumen microbial population dynamics during adaptation to a high-grain diet	2010	ABI 3700	*M. elsdenii*, *S. bovis*, *S. ruminantium*, and *P. bryantii* increased with additional grain in the diet while fibrolytic bacteria decreased.	(Fernando et al. 2010)
Evaluation of bacterial diversity in the rumen and feces of cattle fed different levels of dried distillers grains plus solubles using bacterial tag-encoded FLX amplicon pyrosequencing	2010	454 GS FLX	Abundance of the phylum *Bacteroidetes* increased with addition of distillers grains to growing diet.	(Callaway et al. 2010)
Microbiome analysis of dairy cows fed pasture or total mixed ration diets	2011	454 GS FLX	Pasture diet increased *Prevotella* abundance regardless of fraction. Protozoal community was unaffected by diet or rumen fraction.	(de Menezes et al. 2011)
Metagenomic discovery of biomass-degrading genes and genomes from cow rumen	2011	Illumina Hiseq 2000	88% of carbohydrate-active candidate genes were greater than 25% dissimilar to deposited genes.	(Hess et al. 2011)
Characterization of the rumen microbiota of pre-ruminant calves using metagenomic tools	2012	454 GS FLX	Significant changes in phylogenetic composition of the rumen microbiome occurred during development, but the metabolic potential remained stable.	(Li et al. 2012)
Nitrogen metabolism and rumen microbial enumeration in lactating cows with divergent residual feed intake fed high-digestibility pasture	2012	454 GS FLX	Archaea, bacteria, protozoa, and fungal communities were largely similar between inefficient and efficient cows. Differences were observed for 3 bacterial families and two protozoa species.	(Rius et al. 2012)
High throughput whole rumen metagenome profiling using untargeted massively parallel sequencing	2012	Illumina GAIIx	There is greater variation in the rumen metagenome between animals than replicates from the same rumen.	(Ross et al. 2012)
Composition and similarity of bovine rumen microbiota across individual animals	2012	454 GS FLX	Although significant heterogeneity exists between the rumen microbiome of animals on a similar diet, phylogenetic comparisons indicate more similarities.	(Jami and Mizrahi 2012)
Next generation sequencing to define prokaryotic and fungal	2012	454 GS FLX	Compared with the bacterial community, archeal and	(Fouts et al.

diversity in the bovine rumen			fungal communities were more consistent in the liquid and solid fractions.	2012)
Comparative survey of rumen microbial communities and metabolites across one caprine and three bovine groups using barcoded pyrosequencing and 1H-NMR spectroscopy	2012	454 GS FLX	Microbiome composition is affected by diet, host animal breed, and may be associated with rumen metabolites.	(Lee et al. 2012)
Perturbation dynamics of the rumen microbiota in response to exogenous butyrate	2012	454 GS FLX	Exogenous butyrate infusion increased *Ruminobacter* and *Treponema* populations within the rumen microbiome.	(Berg Miller et al. 2012, Li et al. 2012)
Phage-bacteria relationships and CRISPR elements revealed by a metagenomic survey of the rumen microbiome	2012	454 GS FLX, Illumina GAIIx	Rumen bacteriophages are diverse, unique to the rumen, and likely influence the microbial community.	(Berg Miller et al. 2012)
The bacterial community composition of the bovine rumen detected using pyrosequencing of 16S rRNA genes	2012	454 GS FLX	The core rumen microbiome is affected by rumen development and diet.	(Wu S et al. 2012)
The effect of brown midrib corn silage and dried distillers' grains with solubles on milk production, nitrogen utilization and microbial community structure in dairy cows	2012	454 GS FLX	Inclusion of dried distillers grains increased *Bacteroidetes:Firmicutes* ratio and nitrogen and neutral detergent fiber digestion.	(Ramirez HR et al. 2012)
Evaluation of the ruminal bacterial diversity of cattle fed diets containing citrus pulp pellets	2012	454 GS FLX	Greater inclusion of citrus pulp pellets impacted the rumen microbiome and increased bacilli bacteria.	(Broadway P.R et al. 2012)
Simultaneous amplicon sequencing to explore co-occurrence patterns of bacterial, archaeal and eukaryotic microorganisms in rumen microbial communities	2013	454 GS FLX	- Simultaneous pyrosequencing of bacterial, archaeal, and eukaryotic DNA can efficiently describe the entire rumen microbiome and elucidate potential relationships between microorganisms.	(Kittelmann et al. 2013)
Changes in the rumen epimural bacterial diversity of beef cattle as affected by diet and induced ruminal acidosis	2013	454 GS FLX	Minor bacteria in the epimural community were most affected by acidosis challenge and may indicate acidosis susceptibility.	(Petri et al. 2013)
Investigating the effect of two methane-mitigating diets on the rumen microbiome using massively parallel sequencing	2013	Illumina Hiseq 2000	Identified contigs associated with lower methane production were observed in rumen fluid and feces.	(Ross et al. 2013)
Effect of post-extraction algal residue supplementation on the rumen microbiome of steers consuming low-quality forage	2013	454 GS FLX	Dietary inclusion of post-extraction algal residue increased proportion of *Firmicutes* families *Ruminococcaceae*, *Lachnospiraceae*, and *Clostridiaceae*.	(McCann 2013)
Comparative analysis of microbial profiles in cow rumen fed with different dietary fiber by tagged 16S rRNA gene pyrosequencing	2013	454 GS FLX	*Fibrobacteraceae* and *Ruminococcaceae* decreased in relative abundance with increasing concentrate in a forage-based diet.	(Thoetkiattikul et al. 2013)
Effect of dietary forage sources on rumen microbiota, rumen fermentation and biogenic amines in dairy cows	2013	454 GS FLX	Forage source within TMR affected abundance of *Prevotella*, *Rikenellaceae*, *Selenomonas*, and *Ruminococcaceae*.	(Zhang et al. 2014)
Impact of subacute ruminal acidosis (SARA) adaptation of	2013	454 GS FLX	Induction of SARA decreased *Prevotella*, *Treponema*,	(Mao et al.

rumen microbiota in dairy cattle using pyrosequencing			and alpha diversity and may be linked to increased ruminal lipopolysaccharides.	2013)
Characterizing the microbiota across the gastrointestinal tract of a Brazilian Nelore steer	2013	454 GS FLX	Distinct microbiomes exist in segments of the gastrointestinal tract and likely correspond to the physiological function of each segment.	(de Oliveira et al. 2013)
The effects of a probiotic yeast on the bacterial diversity and population structure in the rumen of cattle	2013	454 GS FLX	High yeast treatment decreased *Prevotella* abundance and ruminal ammonia concentration while increasing propionate production.	(Pinloche et al. 2013)
Microbial ecology of the rumen evaluated by 454 GS FLX pyrosequencing is affected by starch and oil supplementation of diets	2013	454 GS FLX	High starch and oil treatment significantly altered the microbiome by increasing *Prevotellaceae* and decreasing *Ruminococcaceae*.	(Zened et al. 2013)
Relationship between the rumen microbiome and residual feed intake-efficiency of Brahman bulls stocked on bermudagrass pastures	2014	454 GS FLX	Inefficient bulls had greater *Prevotella*, and lower stocking intensity increased microbiome diversity and richness.	(McCann et al. 2014)
Microbial biodiversity of the liquid fraction of rumen content from lactating cows	2014	454 GS FLX	Supplementation of lyophilized and dried yeast increased *Bacillus* abundance but did not impact fermentation.	(Sandri et al. 2014)
Establishment of ruminal bacterial community in dairy calves from birth to weaning is sequential	2014	454 GS FLX	The rumen microbiome is established prior to consumption of solid food, but solid food intake determines microbiome composition.	(Rey et al. 2013)
Potential role of the bovine rumen microbiome in modulating milk composition and feed efficiency	2014	454 GS FLX	*Firmicutes:Bacteroidetes* was positively associated with milk fat content while many taxa were related to residual feed intake phenotype.	(Jami et al. 2014)
Effect of feeding dried distillers grains with solubles on ruminal biohydrogenation, intestinal fatty acid profile, and gut microbial diversity evaluated through DNA pyro-sequencing	2014	454 GS FLX	Greater inclusion of DDG with solubles corresponded to higher biohydrogenation of dietary unsaturated fatty acids and a decrease in phylum *Fibrobacteres*. Diet did not affect abundance of biohydrogenating species.	(Castillo-Lopez et al. 2014)
Taxonomic identification of commensal bacteria associated with the mucosa and digesta throughout the gastrointestinal tracts of preweaned calves	2014	454 GS FLX	Three-week-old calves had greater *Prevotella* in the ruminal epithelium-attached community compared to digesta, and had region-specific microbiomes in the gastrointestinal tract.	(Malmuthuge et al. 2014)
Metagenomic analysis of buffalo rumen microbiome: Effect of roughage diet on Dormancy and Sporulation genes	2014	Ion Torrent	Abundance of bacteria at the domain level and presence of Dormancy and Sporulation genes predominantly associated with the Clostridia and Bacilli taxa belonging to the phyla Firmicutes	(Singh et al. 2014)

3.2 Materials and methods

3.2.1 Isolation of antimicrobial genes in IBERS fosmid metagenomic library

3.2.1.1 Agar based functional screen

Metagenomic clones (2 µl) were pressed gently using sterile pin replicators (Molecular Devices Ltd, Berkshire UK) onto Luria-Bertani agar plates which had 500 µl of pathogens *S. aureus, E. coli* K12*, Sal. Typhimurium, Ent. faecalis* and *List. momocytogenes* (OD_{600}nm =1) spread evenly over the agar and left to dry just prior. Plates were incubated at appropriate temperatures for 24 hours and zones of clearing around the clones were noted as being indicative of clones which have gene inserts encoding antimicrobials.

3.2.1.2 Sequence-based screening of putative antimicrobial positive fosmid clones

Sequencing runs were carried out using the high throughput pyrosequencing Genome Sequencer FLX (454 Life Sciences) in Aberystwyth University. In preparation for sequencing, the selected putative antimicrobial-positive fosmids genomic DNA were extracted as described in Section 2.6.2 and fragmented to 600-900 bp fragments by nebulisation. The sheared fosmids were ligated to molecular barcodes (Multiplex Identifiers, MID, Roche Life Sciences) containing short oligonucleotide adaptors "A" and "B". This was in order to specifically tag each sample in the sequencing run. The MID adaptors included an 11-nucleotide sequence tag on adaptor "A" after the sequencing key (see Excel file 'summary of sequencing results' in attached Compact Disc (CD). It can also be accessed on Figshare; link to Figshare and login details are outlined in the list of 'CD/Figshare content' section). The MID-adaptor ligated DNA libraries were mixed in an equimolar amount. The fosmid MID tagged DNA were then clonally amplified by emulsion PCR (emPCR) by using 13.6 x 10^6 Sepharose beads per emulsion reaction. Emulsions were then broken with isopropanol and emPCR beads

were enriched for template-positive beads, bead-attached DNAs were denatured with NaOH and sequencing primers were annealed. About 790,000 beads with clonally amplified DNA were then deposited on two of four 70 x 75 mm regions of the PicoTiterPlate. The PicoTiterPlate device was then loaded onto the 454 instrument along with the sequencing reagents, and sequences were obtained according to the manufacturer's protocol. An SFF (Standard Flowgram Format) file was obtained for each sample, and nucleotide sequence data and phred-like quality scores were extracted. The reads from each of the pooled libraries were identified by their MID tags by the data analysis software gsAssembler v.2.5.3 (Roche Life Sciences) after the sequencing run.

3.2.1.3 Sequence analysis of positive clones

Following sequence assembly using the default parameters on the gsAssembler software v.2.5.3 (Roche Life Sciences), BLASTN (v2.2.28) on NCBI and BioEdit (version 7.1.11) (Hall 1999) were used to edit and trim the vector sequence from the contigs. VecScreen on NCBI was also used to search the sequences for vector contamination. The open reading frames (ORFs) were determined using the ORF finder programme at (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi July 2013), which also does a BLASTP (v2.2.28) (searches the non-redundant protein sequences database using a protein query) search. All ORFs were retained irrespective of peptide length and those with any homology to antimicrobial genes and/or peptides were further investigated. ORFs encoding peptides $\leq$ 50 amino acids and/or without significant hits on NCBI BLAST were explored as potentially antimicrobial gene and/or peptides (see section 3.2.1.4).

These annotations were confirmed with an analysis of the contigs on the RAST server. Multiple alignments of predicted putative antimicrobial genes and their blastp hits were

performed with ClustalW implemented at http://www.ebi.ac.uk/Tools/msa/muscle/ after retrieval of all protein sequences from 'Batch Entrez' on the NCBI website. Alignments were then imputed into Jalview (Thompson et al. 2006, Waterhouse et al. 2009), a multiple sequence alignment visualization software and SeaView (Gouy et al. 2010) (a graphical multiple sequence alignment editor) respectively. Alignments saved in Phylip format were used to build phylogenetic trees using the RAxML BlackBox (Stamatakis et al. 2008) available at http://embnet.vital-it.ch/raxml-bb/. The generated trees were visualized and saved on http://itol.embl.de/ (Letunic and Bork 2011). Where number of alignments were too few to build phylogenetic trees on RAxML BlackBox, the blast pairwise alignment tool was used.

Signal sequences for peptide cleavage were analysed using SignalP 4.1 (Petersen et al. 2011) using the Gram-negative model. Conserved domains in the amino acid sequences were analysed with Conserved-Domain search on NCBI (MacNeil et al. 2001, Marchler-Bauer et al. 2011, Marchler-Bauer et al. 2015) and the Pfam database (version 27.0, available at pfam.xfam.org). No minimum e-value cut-off was applied as ORFs encoding putative positive antimicrobial genes could be potentially novel.

3.2.1.4 Bioinformatic mining and prediction of antimicrobial peptides

Due to the growing interest in AMPs, several algorithms for predicting antimicrobial activity have been developed (Lata et al. 2007, Wang et al. 2009, Torrent et al. 2011) and used to confirm activity of short AMP sequences (Fjell et al. 2009, Hadley and Hancock 2010). In this study, prediction of antimicrobial peptide sequences within open reading frames (identified using NCBI ORF Finder) was completed using a combination of publicly available databases listed below.

3.2.1.4.1 The antimicrobial peptide database APD

APD is a general multifunctional antimicrobial peptide database dedicated to antimicrobial peptides from all biological sources, ranging from bacteria, plants, to animals, including humans (Wang and Wang 2004, Wang et al. 2009). The database collects only 'mature and active' peptides (<100 amino acid residues), rather than a mixture of mature and precursor proteins. The peptides in APD are classified based on their biological activities such as anticancer, antiviral, antifungal and antibacterial, allowing users to readily obtain a list of peptides of special interest to them. APD provides interactive interfaces for peptide query, prediction and design. It also provides statistical data for a select group of or all the peptides in the database APD is a useful tool for studying the structure-function relationship of antimicrobial peptides. APD is created, maintained and updated by the NMR Structural Biology Group at Eppley Institute, the University of Nebraska Medical Centre and can be accessed via the Internet at the http://aps.unmc.edu/AP/main.html.

3.2.1.4.2 AMPA

The Antimicrobial Sequence Scanning System, AMPA (Torrent et al. 2009, Torrent et al. 2012) is a theoretical approach that predicts antimicrobial proteins from their amino acid sequence in addition to determining their antimicrobial regions. A bactericidal propensity index has been calculated for each amino acid, using the experimental data reported from a high-throughput screening assay as reference. Scanning profiles were performed for protein sequences and potentially active stretches were identified by the best selected threshold parameters. The method was corroborated against positive and negative datasets and active antimicrobial sequences could be spotted from experimental data. AMPA can correctly identify antimicrobial proteins with an accuracy of 85% and a sensitivity of 90%. The method can also predict their key active regions,

making this a tool for the design of new antimicrobial drugs. It is available at http://tcoffee.crg.cat/apps/ampa/do.

3.2.1.4.3. BACTIBASE

BACTIBASE (Hammami et al. 2007, Hammami et al. 2010) contains calculated or predicted physicochemical properties of 177 manually curated annotation of bacteriocins and their sequences produced by both Gram-positive and Gram-negative bacteria. It includes data collected from published literature as well as high-throughput datasets. BACTIBASE allows rapid prediction of relationships structure/function and target organisms of these peptides and therefore better exploitation of their biological activity and could have implications for the development of new drugs for medical use in both the medical and food sectors. The database incorporates various tools for bacteriocin analysis, such as homology search, multiple sequence alignments, Hidden Markov Models, molecular modelling and retrieval. It can be accessed online at http://bactibase.pfba-lab-tun.org/antiinfective.php.

3.2.1.4.4 CAMP

CAMP (Collection of Anti-Microbial Peptides) (Thomas et al. 2010, Waghu et al. 2014) is a manually curated database and currently holds over 6000 sequences and 682 3D structures of AMPs. It was developed for advancement of the present understanding on antimicrobial peptides. The prediction tools for AMPs are based on machine learning algorithms like Random Forests (RF), Support Vector Machines (SVM) and Discriminant Analysis (DA) (Norušis 1988, Vapnik 1995, Breiman 2001, Muller et al. 2001). The prediction models on CAMP gave accuracies of 93.2% (RF), 91.5% (SVM) and 87.5% (DA) on the test datasets. CAMP is a useful database for study of sequence-activity and specificity relationships in AMPs. It is free available online at http://www.camp.bicnirrh.res.in/.

3.2.1.4.5 AntiBP2

AntiBP (Antibacterial peptides) (Lata et al. 2007, Lata et al. 2010) uses a robust method for predicting antibacterial peptides from amino acid sequence of a given peptide. It assesses whether or not they have antibacterial properties based on the observation that certain residues are preferred over others in antibacterial peptides, particularly at the N and C terminus. The database further classifies antibacterial peptides according to their sources with an overall accuracy of 98.95% as well as in their respective families with satisfactory results. The database may be helpful in discovering efficacious antibacterial peptide, which we may help against antibiotics resistant bacteria. It can be assessed online at http://www.imtech.res.in/raghava/antibp2/index.html.

3.2.1.5 Selection of AMPs for further characterisation

Briefly, all predicted ORFs were uploaded into different databases discussed previously. Only those ORFs which were predicted to be antimicrobial peptides in at least two databases were explored further. Because the AMPA server could predict key active regions/stretches within ORFs already predicted as AMPs, it was used as a definitive tool in the final selection of AMPs. This was also experimentally confirmed; One ORF (MRCEI**PHRCVRRKYRIRRHSP**FSCATVGKGDRCGPLRYYASWRKGDVLQGD with 51 amino acid (AA) residues) predicted to be an AMP and the active stretch within it (PHRCVRRKYRIRRHSP) highlighted above with 16AAs- designated SHALININ and SHALININ 1 respectively) were chosen at random and chemically synthesized at >80% purity by GL Biochem Shanghai Ltd. The antimicrobial activities of these peptides were then investigated as described in section 2.5.1. This was to determine whether the full gene (predicted ORF) was required for antimicrobial activity or whether the short predicted active region/stretch within the ORF alone was sufficient for antimicrobial activity. If the former were the case, then traditional cloning and

expression of the genes would be more logical and economical due to the length of the proteins especially when ORFs are longer than 25 AAs. If the latter was to be true, on the other hand, it would be logical to chemically synthesize the active stretches alone for the same reason, and subsequently investigate their antimicrobial activities. In other words, all predicted AMPs were classified as either short (≤25 AAs) or long (>25) proteins/peptides. Results from this preliminary analysis revealed that the predicted active region within the ORF was more active against tested bacteria strains than the full length ORF itself (see Figure 3.10 in CD/Figshare for this data). Therefore, for ease of chemical synthesis, only the active regions in ORFs predicted to be AMPs (especially those which were ≤ 22 AAs), were included in the peptide library generated for further antimicrobial screening. All AMPs >22 AAs were expressed in *E. coli*.

3.2.2 Identification of AMPs from cow rumen metagenome dataset

3.2.2.1 Rationale for choosing the rumen metagenomic dataset used in this study

As can be seen from Table 3.1, most research publications on bovine rumen metagenomic datasets began in 2010. It should also be noted that many of the sequence data were not publicly available at the time of publication but one or two years later. At the time of investigation (July, 2013), the publicly available cow rumen metagenomic datasets were (Brulc et al. 2009, Hess et al. 2011) and other genomic datasets. To date, the (Hess et al. 2011) is by far the largest available rumen metagenomic dataset containing more than 268 gigabases or 1.5 billion read pairs of metagenomic DNA from microbes adherent to plant fiber incubated in cow rumen. *De novo* assembly of these reads resulted in more than 2.5 million predicted open reading frames at an average of 542 bp and 55% predicted full-length genes, with 27,755 putative carbohydrate-active genes and expressed 90 candidate proteins, of which 57% were enzymatically active against cellulosic substrates. In addition, 15 uncultured microbial genomes, which were

validated by complementary methods including single-cell genome sequencing, were assembled. The data set provides a substantially expanded catalog of genes and genomes participating in the deconstruction of cellulosic biomass. This is more than five times greater than the number of candidate carbohydrate genes from any previous study highlighting the benefits of deep sequencing of the rumen metagenome in the definition and discovery of metabolic potential. Consequently, this dataset was chosen and mined for genes with potentially novel antimicrobial activity purely by use of a combination of bioinformatic prediction software/tools. Moreover, it was useful to compare the abundance, if any, of antimicrobial genes within another cow rumen metagenomic dataset and the dataset available from the fosmid based rumen dataset at IBERS.

3.2.2.2 Method for mining rumen metagenomic dataset

All datasets (libraries) used for similarity analysis and antimicrobial peptide prediction and their respective sources are as follows: The Library "Cow" dataset contained predicted protein sequences 'metagenemark_predictions.faa.gz' (2,547,270 sequences) available at (http://portal.nersc.gov/project/jgimg/CowRumenRawData/submission/) (Hess et al. 2011). The Library "AMP1", containing a list of known antimicrobial peptides (AMPs) downloaded from APD2: Antimicrobial Peptide Search database containing 2308 peptides (downloaded on November 10, 2013) (Wang et al. 2009) available at http://aps.unmc.edu/AP/main.php and the Library "AMP2", containing a list of 48 synthetic AMPS found by Kai Hilpert (Ramon-Garcia et al. 2013). The MATLAB toolbox Gait-CAD (Mikut et al. 2008) including the Peptides Extension Package (Mikut 2010) were used for most of the computational analysis. Gait-CAD is free software and can be downloaded from http://sourceforge.net/projects/gait-cad/ but requires the

commercial MATLAB software to work. MATLAB analysis was done in collaboration with Prof Ralf Mikut at Karlsruhe Institute for Technology (KIT), Germany.

The “Cow” dataset was imported using "fastread" function of MATLAB Bioinformatics toolbox. Imported data was split into 26 parts with 100,000 sequences each to make computation easier. Only protein sequences with a maximum length of 200 amino acids (AA) and not more than 5% unknown AAs (marked by X, *) were selected from the “Cow” predicted protein sequences from metagenemark_predictions.faa.gz. The APD2 recommends that small antimicrobial proteins should have a length <200 AAs and most AMPs (>90%) have a length of <60 AAs (Wang et al. 2009). Libraries "AMP1" and "AMP2" were fused to produce Library "AMP" with 2356 peptides. AA distribution and AA dimer (pair) distribution were then computed, resulting in proportion for 20 AAs (and 20x20 = 400 AA dimers) for Libraries "Cow" and "AMP" Libraries. For each candidate of "Cow", distances ("Distance (20)" and "Distance (420)") to each peptide in "AMP" were computed. AA acid distributions with minimal value of 0 for identical and maximal value of 2 for only completely different AAs, were termed "Distance (20)". AA acid and AA acid pair distributions with minimal value of 0 for identical and maximal value of 4 for only completely different AAs and AA pairs, respectively (distance for 400 + 20 features), termed "Distance (420)". For each sequence in "Cow", minimal distance values from previous computational step and number of most similar peptide from "AMP" were saved as separate features. Again, all sequences from "Cow" with small AA distances (Distance (20) <0.2 or a small AA and AA pair distances (Distance (420) <1.45 were saved. All sequences that fulfill the conditions in the previous step were collected and written into Excel (see Table 3.9 "Rumen candidates" in CD/Figshare). Some interesting hits were then used to check similarity in APD2 database (criteria: small distance of AA pairs, different neighbors to explore the variety

of found candidates). Finally, descriptors (Figures 3.7 to 3.9) were computed following procedures described by (Mikut and Hilpert 2009, Mikut 2010).

3.3 Results

3.3.1 Isolation of antimicrobial genes in IBERS fosmid metagenomic library

3.3.1.1 Agar-based functional screen

After incubation at appropriate temperatures for 24 hours, zones of clearing around clones, indicative of gene inserts encoding antimicrobials was observed in 255 out of 8448 clones screened. Figure 3.1 shows an example of a plate with clones showing zones of clearing around them. Table 3.2 below shows a list of all 255 positive clones.

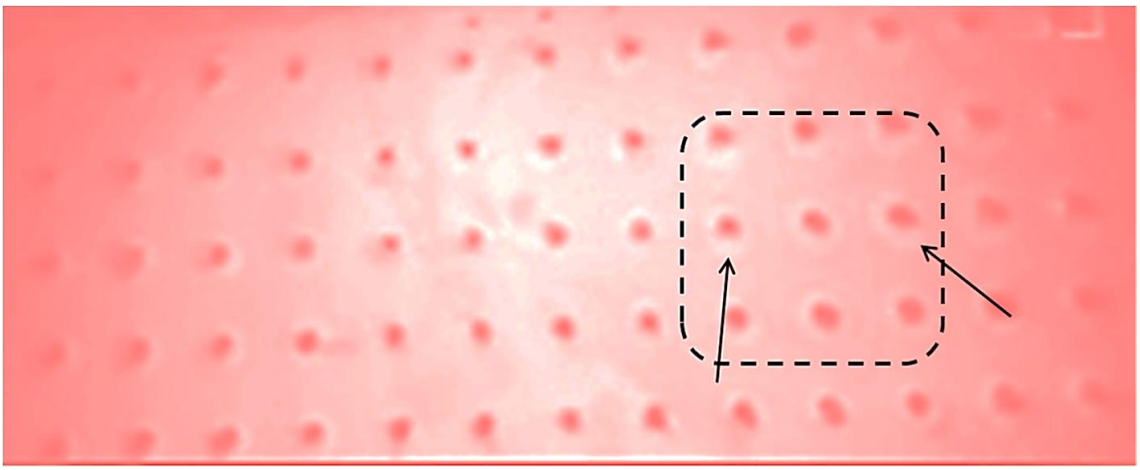


Figure 3.1 Example plate from agar-based screen. Area on plate showing clones with zones of clearing around them is in dashed box and arrows point to some clones with a clearing around them. Screen was done on LB plate. Red colour from a food dye was added to aid visibility.

Table 3.2 Results from agar based screening of rumen metagenomic clone library - showing only clones with growth and activity in ≥3 out of 6 screens. Column 1 is plate ID of the SAB library, columns 2-6, describe the well location on the individual plates, i.e. the specific clone with activity against the different pathogens.

	E. coli										*Sal. Typhimurium*										*S. aureus*							*Ent. faecalis*				*L. monocytogenes*							TOTAL
Fosmid/Plate ID	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	1	2	3	4	1	2	3	4	5	6	7	
SAB PL 1											F13																												**1**
SAB PL 2																																							0
SAB PL 3											H13																												**1**
SAB PL 4	J3	L2	M2	N2	N4	N5					J16	J18	N2																										9
SAB PL 5	C17	F15	G14	I12	I13						B17	C17	E15	I13	I15	J15	L9											A1	A2	B1	B2	K7								**17**
SAB PL 6	A12	A13	L6	M5	M6	N10	P20				P20																												8
SAB PL 7	D19	D20	E19	F20	F23	G24	H8	H13	J5	J20	D19	E19	E24	H12	H13	K22	K23	L20	L21	O5												I11	J9	K11					
	K22	K23	K24	L20	M19	M22	M23	N10	N15	N20	O10	O21	O23	P4	P19	P20	P24																						
	N21	O5	O10	O21	P19	P20	P23	P24																															**48**
SAB PL 8											K5	K6																O1				B10	E20	H17	K13	L14	M15	N14	10
SAB PL 9											N7																	A12				B3	C3	D2	F16	I7	L10		**8**
SAB PL 10											C1	M1	O5																										3
SAB PL 11	N5	N6									A14	A15	A17	A21	N5	N6	O5	O6	P5																				**11**
SAB PL 12 (1/2)	C3	E9	M15								H18	I18																D18												6
SAB PL12 (2/2)	A2	A3	A6								A3	A6	B2	B3	D2	D4	E1	E2	E3	E23	C5											J19							
											F1	F2	F5	G1	G2	G13																							**21**
SAB PL 14											A10	M22	P20	P21	P22																								5
SAB PL 15																																							**0**
SAB PL 16																																							0
SAB PL 17																																							**0**
SAB PL 18											A2	A3	A4	A5	A17	A18	A20	A21	B1	B5	C20	F7	G6																
											B8	B9	B20	B21	B23	D17	E12	F7																					**21**
SAB PL 19											A1	A3	B2	B3	D4	D8	F8	F10	K10	K12	A3	A5	D4	D8	E1	F8	F10												
											M22	N10									J11	K10	K12	M22	N10	O8													**25**
SAB PL 27	F18	G13	G15	G18	G19	I14	L9	L10	L12	L13	F17	F18	G15	G17	G18	G19	I14	I15	I17	K18	G15	I17	M12	O3	H14							H14							
											L9	L10	L12	L13	L14	L17	L18	M7	M11	M12																			
											M14	M15	N17	N18	N19	O3	O4	O5	O8	O9																			
											P7	P11	P12	P23																										**50**
SAB PL 28											B18	C3	P21								B18																		**4**
SAB PL 29											A21	G1	M21	N20							H11	I11	K21																	7
TOTAL	**64**										**139**										**26**							**7**				**19**							**255**

3.3.1.2 Sequence analysis of positive clones

A total of 24 positive clones selected at random for further analysis were sequenced as described in section 3.2.1.2. The selected clones are listed in Table 3.3 below. Table 3.4 is a snapshot on the assembly metrics after shotgun sequencing and assembly on the GS FLX system. It shows the total number of reads, base pairs, GC content, contigs and open reading frames (ORFs) in each clone.

Table 3.3 Positive clones selected for sequencing. Column 1 is plate ID of the SAB library, columns 2-6 describe the well location on the individual plates, i.e. the specific clone with activity against the different pathogens

	Fosmid location on 384 well plates				
Fosmid/Plate ID	***E. coli K12***	***Sal. Typhimurium***	***S. aureus***	***Ent. faecalis***	***List. monocytogenes***	**TOTAL**
SAB PL 1						0
SAB PL 2						0
SAB PL 3						0
SAB PL 4	N2					1
SAB PL 5	C17			A1 A2		3
SAB PL 6						0
SAB PL 7						0
SAB PL 8					L14	1
SAB PL 9				A12	B3 F16	3
SAB PL 10						0
SAB PL 11						0
SAB PL 12 (1/2)	C3	H18		D18		3
SAB PL12 (2/2)	A3	G13			J19	3
SAB PL 14						0
SAB PL 15						0
SAB PL 16						0
SAB PL 17						0
SAB PL 18						0
SAB PL 19			A3			1
SAB PL 27	G18 L13	L10 L17	G15 H14			6
SAB PL 28						0
SAB PL 29		M21	H11 I11			3
TOTAL	6	5	5	4	4	24

Table 3.4 Snapshot on the assembly metrics after shotgun sequencing and assembly on the GS FLX system.

Fosmid/Plate ID	Total Number of reads	Total number of base pairs	Number of contigs	Total length of contigs (bp)	Insert size from chosen contig (bp)	GC content of insert (%)	Number of ORFs in contig
SAB PL4 N2	26413	17318197	1102	2102927	25841	56.2	132
SAB PL5 A1	47599	30986482	691	4325733	67719	52.7	328
SAB PL5 A2	22427	14871695	1218	2450352	38145	51.5	194
SAB PL5 C17	5733	3699135	76	131887	12070	52.1	53
SAB PL12(1) H18	10168	6616004	203	263016	401	43.8	6
SAB PL8 L14	42326	26689339	1258	3662870	8036	47.9	45
SAB PL9 A12	4707	3113068	85	90709	2240	53.6	11
SAB PL9 B3	15899	10345946	387	572057	31665	48.2	160
SAB PL9 F16	10035	6672761	302	448066	13987	51.9	84
SAB PL12(1) C3	6626	4307213	82	121460	27728	52.6	149
SAB PL12(1) D18	10742	6936903	284	392503	8036	47.92	44
SAB PL12(2) A3	32909	21294400	1261	3447744	27125	47.7	168
SAB PL12(2) G13	17172	10597944	885	1449480	16816	51.1	34
SAB PL12(2) J19	26621	16046320	1290	2782997	31708	49.4	39
SAB PL19 A3	18860	11251168	645	992753	5795	53.4	33
SAB PL27 G15	22181	13408286	890	1518463	29772	45.5	61
SAB PL27 G18	14752	9075890	552	817667	24482	39.5	99
SAB PL27 H14	20027	12073019	950	1581893	12018	50.2	68
SAB PL27 L10	14117	8650852	349	514455	36694	51	193
SAB PL27 L13	14336	8770311	353	544607	35970	52.6	182
SAB PL27 L17	17428	10680537	751	1223750	25007	47.5	124
SAB PL29 H11	16143	10004756	648	988569	33998	49.3	176
SAB PL29 I11	21218	12829662	1101	1996994	23851	43.6	118
SAB PL29 M21	13816	8488354	581	842746	1609	56.6	7

After shotgun sequencing on the GS FLX system and sequence assembly, only contigs of clones from which the vector had been trimmed (i.e. contigs carrying the CopyControl™ pCC1FOS™ vector and gene insert- was the largest contig in most cases) were used for further analysis. The largest contig with a ruminal homolog was used in cases where the vector sequence could not be located. Table 3.5.1 to 3.5.24 in CD/Figshare indicates blastn results (showing first 20 hits) on chosen putatively positive fosmid clones. Only the most similar homologs from blastn hits on all clones are outlined in Table 3.6 below.

When homology searches were done on the open reading frames predicted in the selected clones, very low similarity to already known antimicrobial genes and/or gene classes was observed. All predicted ORFs (putative protein-coding genes) in the chosen contig of all 24 clones can be found in 'all open reading frames' folder in CD/Figshare. The blastp results of all ORFs can be accessed on the NCBI, although homologs may differ depending on access date. The blastp results used in this study were saved in order to avoid discrepancies due to website updates (see folder 'blastp results for all ORFs' in the attached CD. Only ORFs of interests, i.e. ORFs with some homology to antimicrobial biosynthetic genes are listed in the Table 3.7 and 3.8 below. Table 3.9 is a list of conserved domains in predicted antimicrobial coding genes/proteins. The phylogenetic trees of predicted antimicrobial coding genes/proteins and their protein homologs can be found as pdf files in Figures 3.2 to 3.6 in CD/Figshare.

Table 3.6 BLASTN hits showing the most similar homologs for all selected clones contigs

Plate/clone ID	Description	Max score	Total score	Query cover	E-value	Identity	Accession
SAB PL4 N2	*Prevotella ruminicola* 23, complete genome	2542	7478	31%	0	92%	CP002006.1
SAB PL5 A1	Uncultured bacterium Contig939 genomic sequence	6739	6739	5%	0	100%	KC246977.1
SAB PL5 A2	*Prevotella ruminicola* 23, complete genome	1581	9168	32%	0	82%	CP002006.1
SAB PL5 C17	*Acinetobacter baumannii* AB0057, complete genome	1819	1819	7%	0	99%	CP001182.1
SAB PL12(1) H18	*Prevotella ruminicola* 23, complete genome	160	160	35%	2.00E-35	86%	CP002006.1
SAB PL8 L14	*Acinetobacter baumannii* AB0057, complete genome	1833	1833	11%	0	99%	CP001182.1
SAB PL9 A2	*Morganella morganii* subsp. *morganii* KT, complete genome	3538	23606	100%	0	94%	CP004345.1
SAB PL9 B3	*Prevotella ruminicola* 23, complete genome	1127	1791	6%	0	81%	CP002006.1
SAB PL9 F16	*Prevotella ruminicola* 23, complete genome	469	670	9%	4.00E-127	77%	CP002006.1
SAB PL12(1) C3	Uncultured organism XynA gene, complete cds	544	544	3%	2.00E-149	76%	JX154664.2
SAB PL12(1) D18	*Acinetobacter baumannii* AB0057, complete genome	1833	1833	11%	0	99%	CP001182.1
SAB PL12(2) A3	Uncultured bacterium Contigcl_1559 genomic sequence	14515	14588	24%	0	99%	KC246861.1
SAB PL12(2) G13	Uncultured bacterium Contigcl_138 genomic sequence	21309	21309	66%	0	99%	KC246851.1
SAB PL12(2) J19	Uncultured bacterium Contigcl_138 genomic sequence	24211	24286	39%	0	99%	KC246851.1
SAB PL19 A3	*Prevotella ruminicola* 23, complete genome	2250	2250	33%	0	87%	CP002006.1
SAB PL27 G15	*Prevotella ruminicola* 23, complete genome	6334	20112	74%	0	87%	CP002006.1
SAB PL27 G18	*Edwardsiella ictaluri* 93-146, complete genome	137	137	0%	1.00E-26	81%	CP001600.2
SAB PL27 H14	Uncultured bacterium Contig1335 genomic sequence	9642	9642	41%	0	99%	KC247026.1
SAB PL27 L10	*Prevotella ruminicola* 23, complete genome	2532	6713	21%	0	80%	CP002006.1
SAB PL27 L13	*Prevotella ruminicola* 23, complete genome	6753	28065	82%	0	82%	CP002006.1
SAB PL27 L17	*Prevotella ruminicola* 23, complete genome	5614	10809	45%	0	81%	CP002006.1
SAB PL29 H11	*Prevotella ruminicola* 23, complete genome	1377	4261	12%	0	89%	CP002006.1
SAB PL29 I11	*Coprococcus* sp. ART55/1 draft genome	548	737	5%	1.00E-150	75%	FP929039.1
SAB PL29 M21	*Prevotella ruminicola* 23, complete genome	931	931	99%	0	77%	CP002006.1

Table 3.7 Predicted antimicrobial (biosynthetic) protein coding genes in fosmids. All ORFs are from contig 1 of each fosmid and are in the 5'-3' direction.

Fosmid Plate ID/ORF	Given name	Protein size (aa)	Putative function	Most similar homolog (e-value)	Identity (overlapped aa)
SABPL5 C17/11	Gene 6	184	**4'-phosphopantetheinyl transferase family protein** synthesis of unusual molecules including polyketides, atypical fatty acids, and antibiotics	*Prevotella ruminicola* 23 WP_013063463.1 (3e-104)	140/184(76 %
			putative biosurfactants production protein	*Butyrivibrio crossotus* CAG:259 WP_021960962.1 (2e-33)	58/161(36 %
SABPL12(1) C3/9	Gene 17A	350	**3-dehydroquinate synthase** DHQS represents a potential target for the development of novel and selective antimicrobial agents	*Prevotella* sp. CDD20257.1(0.0)	250/346(72 %)
SABPL12(1) C3/50	Gene 17B	80	**colicin V** production protein	*Pseudomonas putida* S16 NP_744149.1 (1.4)	19/61(31%)
SABPL27 L10/66	*palG*1	71	**penicillin** amidase Penicillin Biosynthesis and Metabolism	*Streptomyces mobaraensis* WP_004942604.1 e-value 5.0	16/43(37%)
SABPL27 L10/73	*palG*2	68	**beta-lactam antibiotic** acylase Penicillin Biosynthesis and Metabolism	*Ornithinibacillus scapharcae* YP_004810705.1 e value 8.4	22/63(35%)

Table 3.8 Nucleotide and protein sequences for predicted antimicrobial genes.

Gene name	Nucleotide sequence	size (bp)	Protein sequence	Protein size (aa)
Gene 6	ATGATTACTCTGGTGCGTGAGGATATTTGGGCGTTTGATCTCCAGGCGGCGCTGAAGGAA ATTTCGGAGCAGCGGAGGGAACAGGCGCTGAAGTTCAAACATGAACAGGGGCAACGGCT CTGTGTGTTGGCGTATCTTTTGCTGAAGCAGGCGCTTCGTGAGGGCTACGGTATCACAGA GAATCCTGTCTTTGAATATAATGAGCACGGTAAACCCTCGATCGTGGGACATCCGGAAAT CTTCTTCAATCTGAGTCATTGTAAGGAGGCTGCCATCTGTGTGGTGAGCGATCAACCTGTG GGTGTCGATGTGGAGGGTATCCGCGAATATAAGGAGTCGTTGGTGAACTATACGATGAAT GACGAGGAGATCGCTCAGATCAAGACGGCAGAGAATCCTGCCGCTGCCTTCATCCGCTTA TGGACGATGAAGGAGGCTACCACCAAACTGATTGGTACGGGCATCAGCAACGACATGAA AACCGTGATAGACCCCACTAAATATAAATATACCACTGTCGATCGACAGCGGTATATTTA CACCATATGTGAATAG	555	MITLVREDIWAFDLQAALKEISEQRREQALKFKHEQGQR LCVLAYLLLKQALREGYGITENPVFEYNEHGKPSIVGHPE IFFNLSHCKEAAICVVSDQPVGVDVEGIREYKESLVNYTM NDEEIAQIKTAENPAAAFIRLWTMKEATTKLIGTGISNDM KTVIDPTKYKYTTVDRQRYIYTICE	184
Gene 17A	ATGGATAAACAGAAGGTTATCATATCGCTCCAGTTGGAGCCGACGCTCGCCTCAGCCGTT GCTGAATGTGAGCGCGACCGCATCTTCATCTTGGTTGATGAAACGACGGAAAAGCTCTGT CTGCCGCTGATTGCAGGCTTTGACTGCGTGCGCGAGGCCGAGATCATTACTATTGGTGCC ACCGATCAGCACAAGACGCTTGACTCGCTGAGCCACGTCTGGTCGTGCCTGCAGCAGGGT GGGGCCACCCGCCATTCGCTGATGGTGAACCTGGGCGGCGGCATGGTGACCGACCTGGGC GGCCTTGCTGCCTCAACCTTCAAGCGCGGCCTGAACTACATCAACATTCCCACCACGCTGC TCTCGATGGTCGATGCCTCCGTAGGCGGCAAGACGGGCATCAACTTCGGCGGACTGAAGA ATGAAATCGGCGTGTTCAACAATGCCCGCTCAGTGATTCTCGACACCACGTTCCTCAGGA CGATGGACTACGAGAACATCTGCTCAGGCTATGCCGAGATGCTGAAGCACGGCCTGATAG CCACCGACGACATGCTGGACGAGCTGTTGGCGTTCGACCTCGACGTCATTGACTATTACC ACCTGCAGCGTATGGTGGCCGACTCGGTGAAGGTGAAGGAAGGCATCGTAGAGAAAGAC CCcACGGAACGAGGCATCCGCAAGGCACTCAACcTGGGACACACCGTAGGCCACGCTTTC GAGTCGCTGGCTCTGCAGCGCAAGCCCGTGCTCCATGGTTATGCGGTGGCCTGGGGACTT ATCTGCGAACTCTACCTTAGCTGCATCAAGACGGGGTTCCCAGTCGAACGGATGCGGCAG GCTGTCCGCTTTATCAGTGAGCACTACGGCAAGATGCCCATCACCTGCGACGACTATCCC ACGCTGCTGGAACTGATGACCCACGACAAGAAGAACGTGGCAGGACAGATTAACTTCAC GCTGCTGGGCGCGGTGGGCGATATTCGTATCAACCAGACGGCCACGCAGGAAGAGATAC AAGAAGCCCTCGACTTTTATCGCGAGGGGAACTAA	1053	MDKQKVIISLQLEPTLASAVAECERDRIFILVDETTEKLCL PLIAGFDCVREAEIITIGATDQHKTLDSLSHVWSCLQQGG ATRHSLMVNLGGGMVTDLGGLAASTFKRGLNYINIPTTL LSMVDASVGGKTGINFGGLKNEIGVFNNARSVILDTTFLR TMDYENICSGYAEMLKHGLIATDDMLDELLAFDLDVIDY YHLQRMVADSVKVKEGIVEKDPTERGIRKALNLGHTVG HAFESLALQRKPVLHGYAVAWGLICELYLSCIKTGFPVE RMRQAVRFISEHYGKMPITCDDYPTLLELMTHDKKNVA GQINFTLLGAVGDIRINQTATQEEIQEALDFYREGN	350
Gene 17B	ATGGGCTGTTCTATCGAGTGGATGTTGTATTGCGACAGTAGTTCCAGCTTGTACAGTGCCT CTTCGAACGGGAAGGCCCCGTTGGCATCGAGCCGCAGTTCCACCTCATGGAACGAGAAAC GGTTGCGGATGCACTTCACCAGACTCAGTTCCTGGTCAAAGTCGATGGCCCCGATTTTCAa CTTCACACAGTGGAAGCCCTGCTCCAGCTTCTGTTCCAATCGTGCAAGCATCTCGTCGTAG	243	MGCSIEWMLYCDSSSSLYSASSNGKAPLASSRSSTSWNE KRLRMHFTRLSSWSKSMAPIFNFTQWKPCSSFCSNRASIS S	80
*palG*1	ATGAGGCTGTCACACGTTTGTAGTCAGAGTTCTTCAAAGTACCCTGCTGGTTCGTGTAACC TAATGACACACGATAGGGCAACTTCTTCAGAGCACCGGTCAGCGTCACGTTGTGATCGTG TGATACGGCGGTTCTGTAGATCAGATCCTGCCAGTCGGTATTGGCGGTACCCAGTGCGCG GTATGCGTCATGGTCGGTGCCATACAGATTGGTGA	216	MRLSHVCSQSSSKYPAGSCNLMTHDRATSSEHRSASRCD RVIRRFCRSDPASRYWRYPVRGMRHGRCHTDW	71
*palG*2	ATGAGCGCCATGAGGCACGATGTACTTCCTCACGTTGGGATTCGTGGGATCGGGAATGGC ACCACCCGTCCAGGGATCATCCTCGCCATAGACGAAGATCATCTTCTTATTGGTGGTCTCC AGATTCTTCAGCAGCTTCACCTGCAGATCGATCGTCTTGGTGTTTCTGCTGTCCTGCATGG CATCCTTGAAATATGCCTTGTCTGA	207	MSAMRHDVLPHVGIRGIGNGTTRPGIILAIDEDHLLIGGL QILQQLHLQIDRLGVSAVLHGILEICLV	68

Table 3.9 Conserved domains in predicted antimicrobial coding genes and proteins

Protein	Conserved domains				
	Name	Accession	Description	Interval on native protein	E-value
Gene 6	ACPS	pfam01648	4'-phosphopantetheinyl transferase superfamily	99-162	1.02E-09
	pantethn_trn	TIGR00556	phosphopantetheine--protein transferase domain	96-157	2.37E-03
	AcpS	COG0736	Phosphopantetheinyl transferase (holo-ACP synthase) [Lipid metabolism]	100-157	2.92E-03
	Sfp	COG2091	Phosphopantetheinyl transferase [Coenzyme metabolism]	21-156	1.08E-22
	PRK10351	PRK10351	holo-(acyl carrier protein) synthase 2	62-149	3.65E-13
Gene 17A	DHQS	cd08195	Dehydroquinate synthase (DHQS) catalyzes the conversion of DAHP to DHQ in shikimate pathway ...	6-343	1.61E-128
	aroB	TIGR01357	3-dehydroquinate synthase	24-344	4.88E-101
	aroB	PRK00002	3-dehydroquinate synthase	6-344	1.18E-99
	DHQ_synthase	pfam01761	3-dehydroquinate synthase	55-312	6.08E-98
	AroB	COG0337	3-dehydroquinate synthetase [Amino acid transport and metabolism]	13-344	2.07E-96
	PLN02834	PLN02834	3-dehydroquinate synthase	26-346	3.98E-52
	PRK13951	PRK13951	bifunctional shikimate kinase/3-dehydroquinate synthase	65-328	9.99E-47
Gene 17B	No conserved domain found				
*palG*1	No conserved domain found				
*palG*2	No conserved domain found				

3.3.1.3 Bioinformatic mining and prediction of antimicrobial peptides

As previously stated, all ORFs were uploaded into different AMP predicting databases. Only the active regions in ORFs predicted to be AMPs on the AMPA server were

included in the peptide library generated for further antimicrobial screening. A total of 181 short AMPs (predicted active regions in ORFs) were identified (see file 'all AMPs' in CD/Figshare). However, based on length and for ease of synthesis, only 135 of these AMPs (≤22 AAs) were included in the peptide library created. Peptide library synthesis was carried out as described in section 2.7. The comprehensive list of all 135 peptides included in peptide library can be found in the file 'peptide library' on CD/Figshare.

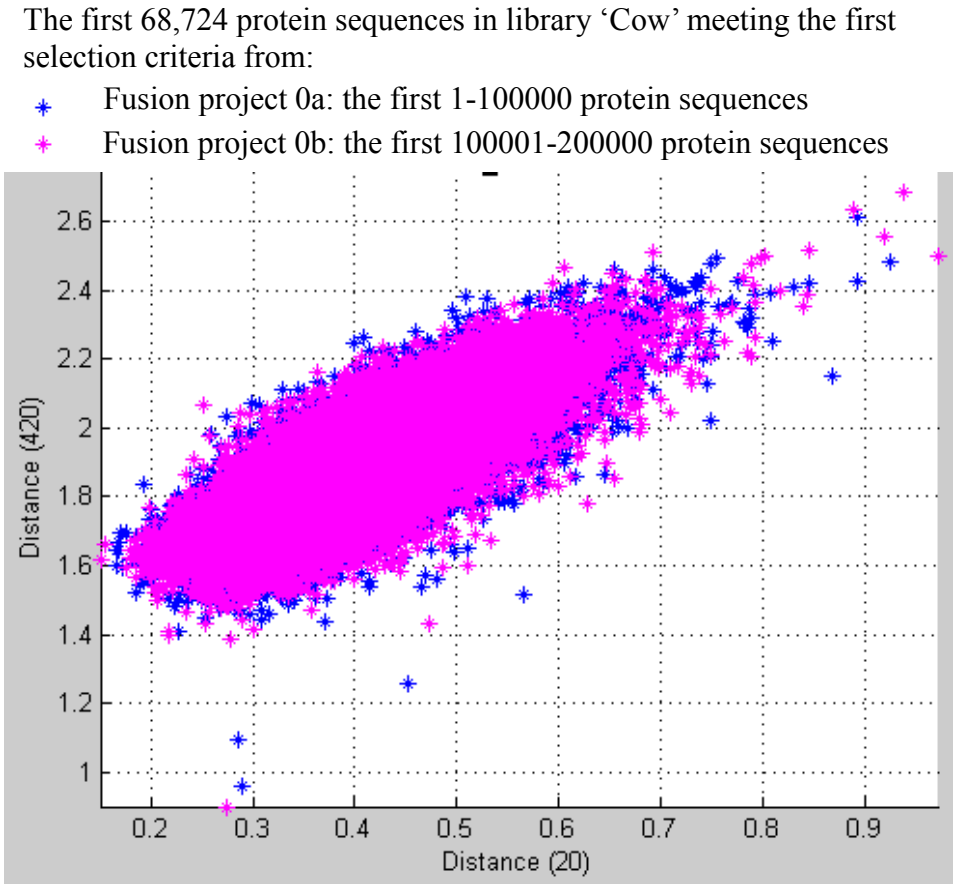
3.3.2 Identification of AMPs from the rumen metagenomic dataset

3.3.2.1 Antimicrobial genes predicted from the rumen metagenomic dataset using computational analysis

A summary of results from each computational step is summarized in Table 3.10 below. There were 917,636 sequences remaining after the first selection criteria (i.e., only protein sequences with a maximum length of 200 amino acids (AAs) and not more than 5% unknown AAs (marked by X, *) were selected from the "Cow" predicted protein sequences). Out of these 917, 636 sequences, only a few sequences (829 sequences in total) fulfilled the criteria of AA distances (Distance (20) <0.2 or a small AA and AA pair distances (Distance (420) <1.45 (see Table 3.11.1 "Rumen candidates" in CD/Figshare). For example, only 65 sequences met these criteria in the first 68,724 sequences with isolated points outside a relatively dense distribution as illustrated in Figure 3.7.

Table 3.10 Summary of results from computation steps

Fusion project	First sequence number	Number of sequences	Number of sequences after first filtering	Candidates fulfilling distance criteria
0a	1	100000	35027	32
0b	100001	100000	33247	33
1a	200001	100000	32561	27
1b	300001	100000	37820	37
2a	400001	100000	39000	32
2b	500001	100000	38806	42
3a	600001	100000	32499	35
3b	700001	100000	28537	19
4a	800001	100000	28701	29
4b	900001	100000	33811	11
5a	1000001	100000	34135	35
5b	1100001	100000	38599	42
6a	1200001	100000	31472	29
6b	1300001	100000	35397	21
7a	1400001	100000	38893	26
7b	1500001	100000	40084	30
8a	1600001	100000	40459	40
8b	1700001	100000	40718	41
9a	1800001	100000	40593	22
9b	1900001	100000	40600	23
10a	2000001	100000	40460	36
10b	2100001	100000	40688	46
11a	2200001	100000	34674	41
11b	2300001	100000	31152	31
12a	2400001	100000	33339	36
12b	2500001	47270	16364	33
SUM		**2547270**	**917636**	**829**



`Figure 3.7 Visualization of distances for AA acids Distance (20) and AA pairs (Distance 420) for the first 68,724 sequences from library "Cow". Candidates with Distance (20)<0.2 or Distance (420)<1.45 are selected as candidates (here: 65).

Although, it was difficult to rate the probability that these sequences are antimicrobial, six most promising sequences (termed Hess-AMP gene 1-6 (H-G1- H-G6)) were identified out of the 829 sequences, (marked yellow in Table 3.11.1 in CD/Figshare). They are also summarized in Table 3.11 below. Different APD2 hits were also identified for these 6 AMP sequences, also shown in Table 3.11 below. Table 3.12 provides the corresponding nucleotide sequence and other information on the identified genes. These can be accessed from the file "cow_rumen_initial_velvet_assembly_scaffolds.fas.gz" found at the following URL http://portal.nersc.gov/project/jgimg/CowRumenRawData/submission/).

Table 3.11 Six of the most promising antimicrobial peptide sequences (H-G1-H-G6) identified in cow rumen metagenome dataset

Assigned name	Sequence	Amino acid (AA) (length)	Location on cow dataset	Most similar homolog on APD2 (stop codon '*' removed)	
				APD ID	Similarity %
H-G1	VKKAPAKKAAPKAAAKENVKAAAAPATKSTKKPKKV*	37	NODE_3603970_length_237431_cov_15.909123_orf_36410 39090..39200	AP00537	45%
H-G2	MKKLLLILFCLALALAGCKKAP	22	NODE_664976_length_19740_cov_2.033485_orf_00810 19724..19789	AP00494	40%
H-G3	LLFLALSLLFQLLFPLFLFAFEQQALPFLFLLPLFLLASFQLPLSLFFQLLL*	53	NODE_4144838_length_4700_cov_0.195319_orf_38300 3..161	AP01999	30.76%
H-G4	VLGLALIVGGALLIKKKQAKS*	22	NODE_3958153_length_85376_cov_8.525382_orf_203250 82784..82849	AP01737	48%
H-G5	LFGGGGGTGGFGLGGPGGNGPGGGGGPGGNVPGS*	35	NODE_793869_length_3085_cov_43.351379_orf_34140 complement(2443..2547)	AP02183	52.50%
H-G6	MLSFLFMQKLMKKKLKRRKKLKLKRLLLKKKLLKKQLKRFLKRKFL*	47	NODE_2576652_length_4557_cov_3.402019_orf_04080 350..490	AP01010	36.17%

Table 3.12 Scaffold nucleotide sequences from which putative antimicrobials H-G1- H-G6 were identified

Gene ID	Nucleotide sequence on scaffold from which genes have been identified (the highlighted are the regions of interest)	Position on scaffold/orientation	Corresponding Protein sequence	Notes
H-G1	ATGGGATTTCGTGAATCATACAGGCGAGATTCACGACTGGAACAGC CACGGTACTCACTGCGCAGGTATAGCAGCCGCCNNNNNNNNNNGGC CGAGAAGAAAGAG**GTGAAGAAAGCCCCGGCAAAGAAAGCCGCAC CTAAGGCAGCAGCTAAGGAAAACGTGAAGGCTGCAGCAGCTCC GGCTACAAAGTCGACAAAGAAGCCGAAGAAAGTGTAG**	39090..39200 /5'-3'	VKKAPAKKA APKAAAKEN VKAAAAPAT KSTKKPKKV *	part of a larger gene but contains a start and stop codon
H-G2	TCAAGCATCAACATGCTGGAAGCCAGCGCCCTCATGAGCGTCGGCA AGATCGACGAGGCGGAAAGCAAGCTTGACGCCGTCTTCGGGGACGC CAACACCACCGTCGACCAGAAGCAGAGGATCTTGCTTACCAAGGCC ATGCTCGCCCTGCGCGCCAAAGGCGACTCCGAGAGCTTCGAGAAGC TGACGGAAGAAGCCATCGCCCTGGATCCCGAAAGCGAGCTGAGCAA GCAGGCTACCGTCCAGCTGGAGACGGTCAAGAACATGATCAAGGAA AGCAAGCAGGAGCAGGAAAAGGAAGTCGAGCAGGAAGTCAGCGAG GAACAGGAAGAAGCCATAAAGGAACCGGCCGAATAAGCCGAATAC TGTCAGGTCCCAAGC**ATGAAAAAACTTTTGCTCATTCTTTTCTGCC TCGCCCTGGCCCTGGCCGGCTGCAAAAAGGCCCCG**	19724..19789/3'-5'	MKKLLLILFC LALALAGCK KAP	on complementary strand of scaffold sequence
H-G3	**CTTCTCTTTCTTGCTCTTTCTTTGCTTTTTCAGCTTCTTTTTCCTC TTTTTCTTTTTGCTTTTGAGCAGCAAGCTCTGCCTTTTCTCTTTCT TTTGCCTCTTTTTCTGCTTGCTTCTTTTCAGCTTCCTCTCTCT TTATTCTTTCAGCTTCTTCTCTAG**	38300...3..161/ 5'-3'	LLFLALSLLF QLLFPLFLFA FEQQALPFLF LLPLFLLASF QLPLSLFFQL LL	No start codon
H-G4	ATGGACCATCCCTGCCGACCAGAAGCAGCATGCCCGNNNNNNNNNN NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNGGGCGTGGCGACGGTCGCGCATACCT TCCAGGAAGCTTTGAAGGGTGGCAAGACGAAGGTGCTCGATACGCG T AAGACGGTTCCCGGTTTCCGCACGCTGCAGAAGTATGCGGTACGT GTGGGCGGCGGATCCAACCACCGTATGGGCCTTTTCGACATGGTG C	82784-82849/5'-3'	VLGLALIVG GALLIKKKQ AKS*	part of a larger gene but contains a start and stop codon

	TTGTGAAGGACAATCACATTGCTGCGGCGGGTGGCGTGCTCCAG GC GCTCGAAGTGGTGAAGAAGAACAACAAGCAGAACCTGATNNNNNNN NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN NNNNNNNNTTGGGTCGTCGTGTCTTGAAGTTGCGCAAGTGCCTGGA ATGTTTGCCAGAAGGTTTNNNNNNNNNNNT**GTGCTTGGCCTTGCACT** **GATTGTCGGTGGAGCTTTGCTTATCAAGAAGAAACAGGCGAAAT** **CTTAATTTGCGCCATCACGATGGCGACCATCATAATNNNNNNNN** **NNAAGCGCGCTTCTTTCGTTCGTGCTTGGCCTTGTGCTGATTGT** **CGGTGGAGCCTTGCTCATCAAGAA**GAGGCAGGCGAAATCTTAATT TGCGCCATCACGATGGCGACCATCATAATGGCACAACCGCAAATCTC GCGGGCTGAAAGCTGTTCTCCTAGAATGGCCCAACCCGCAAGGACG GCGAATACCGATTCCAGACTCATCGTGAGTGAGGCGATGGTGGGGT TGATTTTATCT			
H-G5	ATGATATCGTTAAATATCTTTACGCTTCTTTGACGCTTATATACTTCC ATAATACCGCGAACAGGATGTCTAGGTGTAGGCCAAATACTTAATTC TTTTTCCTTATCGTTATATCTTACCTGATATGCTTCACCGAAATCAAG CTTTGCTTGTTCAAGCCACATAAGAGCTGCATTCCAGTTACCGAGAA CATCACCGAAACCTGCAGAGTTTCCATAGCATGCGCCACGGAATATA CTGGAAGTATTCATACTCATGACGGAATCATACAATGCATTATGCGG	2443-2547/3'-5'	LFGGGGGTG GFGLGGPGG NGPGGGGGP GGNVPGS	on complementary strand of scaffold (full)

	CAATGTGAAGAGTTCATTGATATCGCCAATCCAGTTTGCGGTCTGGA AATCGATAACTTCTTCAAGTTCTTGGCAAATCTTATAATGCGTCCTGC CAGGTTGCAATTCCATTACAAGATAGTCACGATAGTTACCCTGTCGC GCATAATATCTCTGCACATATCGAATCATGTCGGCGATGATGTAATT TAATTGTTCGTCACTGATTTCAATACAGATTACAGGAGCGCCGAGCA TTAACTTTATATAGTTACGCATTTGCGCCATATTCTGTATCATCGTAG ATTCGAGTACAGGGTCGCAA**TTGTTCGGCGGCGGAGGTGGAACA** **GGCGGTTTTGGCCTTGGCGGACCCGGTGGAAACGGACCCGGCG** **GAGGAGGCGGTCCTGGCGGAAATGTGCCTGGTTCATAG**CCCGGA GGAGGCGGTTTCAATCGTGACCTTGAACCGGTCGGCGGCATTATAGG ATCACGCATAGGCATTAAATTATCGTCAATTACATCACTCAT			
H-G6	**ATGCTATCATTCCTATTCATGCAGAAGCTGATGAAGAAGAAGTT** **GAAGCGGAGGAAGAAGTTGAAGCTGAAGAGGCTCCTGTTGAAG** **AAGAAGCTGTTGAAGAAGCAGTTGAAGAGATTCCTGAAGAGGAA** **GTTTCTTTAG**	350-490/5’-3’	MLSFLFMQK LMKKKLKRR KKLKLKRLL LKKKLLKKQ LKRFLKRKF L*	full gene

Descriptor computations yielded both positively and negatively charged hydrophobicity-loading plots which show that “Cow” sequences (metagenmarks0 to 12) are represented in only a small portion of all AMP regions (blue opaque squares- amp and Hilpert library) (see Figures 3.8 and 3.9).

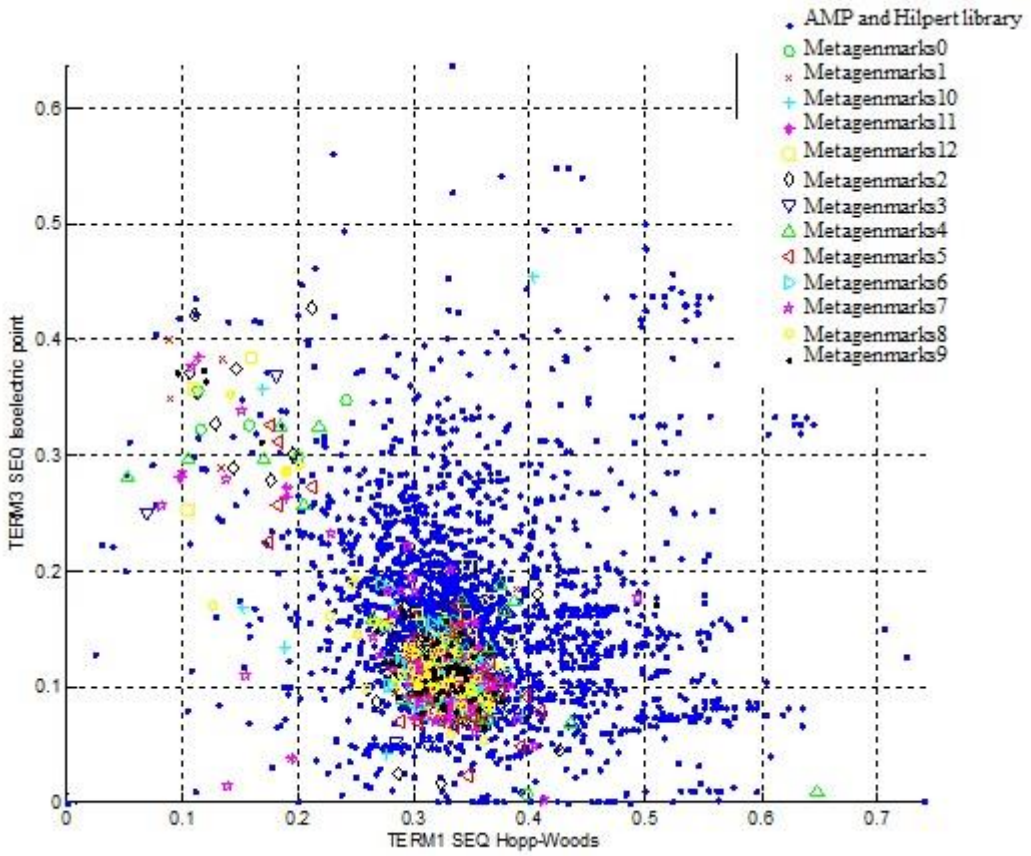


Figure 3.8 Standard hydrophobicity - loading (positively charged), blue dots are known AMPs (library "AMP"), all other colored signs are hits from library "Cow" as described in Table 3.10.

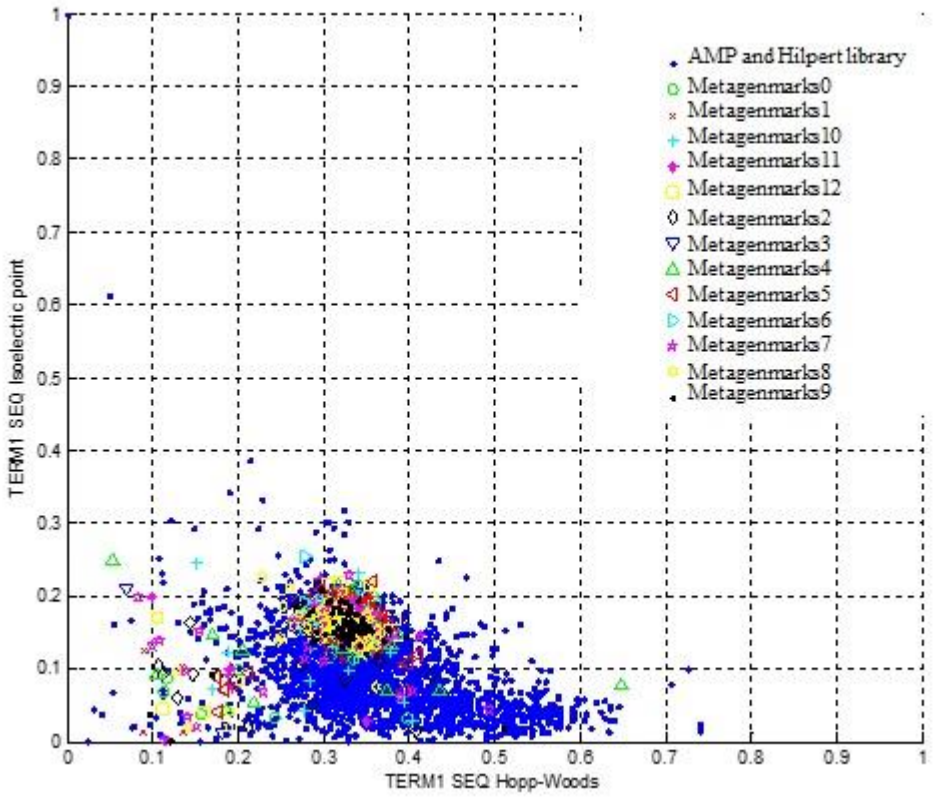


Figure 3.9 Standard Hydrophobicity - loading (negatively charged) plot, blue dots are known AMPs (library "AMP"), all other colored signs are hits from library "Cow" as described in Table 3.10.

3.4 Discussion

Functional genomics is a widely used tool for the discovery of novel bioactive compounds including antibiotics (Wang et al. 2000, MacNeil et al. 2001, Brady et al. 2002, Handelsman 2004). Antimicrobial compounds have been identified in many functional genomics studies using CopyControl (pCC1FOS™ Vector) *E. coli* based fosmid clone libraries (Brady et al. 2002, Burke et al. 2007, Penesyan et al. 2013) similar to the IBERS fosmid based metagenomic library used in this project. Clones are usually screened in an overlay using various microorganisms as target strains.

In this study, it was observed that a number of clones had activity against several target bacteria strains tested including *E. coli* K12 strain, the parent strain from which the recombinant TransforMax™ EPI300™-T1^{R} *E. coli* strain used in fosmid library creation was derived. This raises the question of how activity against *E. coli* K12 could be observed in a recombinant *E. coli* background without toxicity to the host itself. This may be possible as the fosmid clones were not induced to a high copy number during screens and encoded genes may have been expressed in levels not toxic to the heterelogous host. Unless induced to a high copy number (10 to 50 per cell) through the addition of expression inducers (i.e., arabinose or IPTG [isopropyl-β-D-thiogalactopyranoside]) to the growth medium (Penesyan et al. 2013), CopyControl fosmid clones grow at single copy to ensure insert stability and successful cloning of encoded and expressed toxic proteins and unstable DNA (Park et al. 2008). Additionally, in some cases, a zone of clearing could be observed around spots where the clones have been stabbed, even when the clone appeared not to have grown (results not shown). In these cases therefore, it is possible that the encoded genes had killing activity against both the host *E. coli* strain and the target *E. coli* strain tested. This toxicity to host is a common problem in the heterologous expression of antibiotic genes (Uchiyama and Miyazaki 2009, Kimelman et al. 2012) and partly accounts for low discovery rate typically observed in metagenomic screens as is the case in this study. This may also explain the difficulty encountered during expression of proteins discussed in Chapter Four.

Furthermore, many clones in the agar based screen still had a zone of clearing, even in the absence of expression inducers. This may indicate that more antimicrobial gene hits could have been found, if screens were carried out in the presence of an inducer. However, this could also mean that the target *E. coli* strain, and not the genes from the

fosmid inserts were responsible for the antibacterial activity observed (Yung et al. 2011). Nonetheless, the genes identified in this study were verified by blast and PCR to be of ruminal DNA origin (see Figure 3.11 in CD/Figshare).

In terms of the results from the IBERS based metagenomic library, the hits of about 50% of the nucleotide sequences in clones indicative of encoding antimicrobial gene inserts were related to *Prev. ruminocla* 23, 29% were related to various uncultured rumen bacteria and the other clones were related to various rumen bacteria, including *A. baumannii, Morganella morganii*, *Edwardsiella ictaluri* and *Coprococcus* sp. Recent publications of *Prev. ruminocla* 23 genome (Purushe et al. 2010) may account for why most of the fosmid sequences were similar to this entry. Moreover, *Prevotella* has been shown to be one of the more dominant genera in the rumen (Stevenson and Weimer 2007, Bekele et al. 2010, Kong et al. 2010). The high similarity to uncultured rumen bacteria confirms the evidence that many rumen bacteria are yet to be cultured and need to be further explored (Creevey et al. 2014).

When homology searches were done on the ORFs predicted in the selected clones, very low similarity to already known antimicrobial genes and/or gene classes was observed. This could be because antimicrobial activities observed in the agar-based assay are encoded by putatively novel genes not previously annotated to having antimicrobial function. In total, five putative genes (Genes 6, 17A, 17B, *palG*1 and *palG*2) showing similarity to known antimicrobial genes were retrieved in addition to predicted antimicrobial peptides (AMPs). The overall number of genes may however, be underestimated in this study since 454 sequencing results did not allow for the closure of all fosmids and further sequencing could not be repeated due to time constraints. A total of 181 antimicrobial peptides, again with low similarity (<50%) to known AMPs were predicted in all open reading frames using different AMP databases.

Predicted genes included a 4'- phosphopantetheinyl transferase family protein which is involved in the synthesis of unusual molecules including polyketides, atypical fatty acids, and antibiotics, one 3 dehydroquinate synthase, a potential target for the development of novel and selective antimicrobial agents, one colicin V (bacteriocin) production protein, and two penicillin amidase/acyclase genes which catalyse the hydrolysis of benzylpenicillin to phenylacetic acid and 6-aminopenicillanic acid (6-APA) a key intermediate in the synthesis of penicillins. Genes 6 and 17A have conserved domains matching the putative functions of their most similar homologs. Genes 17B, *palG*1 and *palG*2 did not have any conserved domains. None of the five proteins had a predicted signal sequence and/or cleavage position. Phylogenetic analysis of the five proteins and their protein homologs again revealed a distant relationship between the sequences. It was impossible to build a phylogenetic tree to include all five protein coding genes as they were quite dissimilar in size and sequence.

On the other hand, very few antimicrobial genes (6) were predicted in the cow metagenomic dataset, probably due to the stringent parameters used for prediction. Also only AMPs from two datasets were used for homology searching and prediction, as it was challenging to pull out antimicrobial genes from the NCBI and other protein databases, as one would need to search for each antimicrobial gene individually to obtain its sequence before a homology search is performed. Furthermore, a search for antimicrobial genes very readily provides antimicrobial resistance genes instead. It is likely that more antimicrobial genes hits would be identified if more databases were included in the analysis. This highlights the need to create a single and more comprehensive repository/database where all antimicrobial genes are stored especially for ease of prediction and drug design purposes, instead of several currently available databases with limited coverage.

In summary, all predicted AMPs were classified as either short (≤25 AAs) or long (>25 AAs) proteins/peptides. Five longer antimicrobial genes or miniproteins >25 AAs in length (Gene 6, 17A, 17B *palG*1 and *palG*2) including 181 short antimicrobial peptides ≤25 AAs were identified from the in-house metagenome library. Six genes H-G1 to 6 were also identified from the rumen metagenomic sequence dataset. The low similarity of these interesting antimicrobial genes hits to documented sequences in public databases may indicate their novelty. Experimental evaluation to determine the antimicrobial properties of these putative antimicrobial proteins and peptides (AMPPs) is necessary to ascertain their applicability in the treatment of bacterial infections. To investigate the antimicrobial properties of these genes, DNA amplification, expression and purification (or chemical synthesis of shorter AMPs ≤22 AAs) and characterization is required. This is discussed in detail in Chapters Four and Five of this thesis.

CHAPTER FOUR

Expression purification and characterisation of recombinant antimicrobial proteins

4.1 Introduction and aim of chapter

Novel antimicrobial compounds, especially antimicrobial peptides (AMPs) have been regarded as a potential solution to the worldwide emergence and rapid horizontal spread of antibiotic-resistant traits in bacteria of (human and veterinary) clinical significance (Ingham and Moore 2007). They have also been considered as one of the most promising alternative antibiotics because of their strong antimicrobial activity and microorganism eradication with less likelihood of resistance developing (Shin et al. 2013). Therefore, Herculean efforts to develop this new class of antibiotics with novel targets and modes of action have attracted a large amount of attention (Makovitzki et al. 2006). However, in comparison to most antimicrobials, the high manufacturing cost of synthesizing AMPs has hindered their therapeutic application. This has led to the isolation, purification and characterisation of a large number of antimicrobial peptides during recent years using recombinant production of AMPs (Guerreiro et al. 2008, Zorko et al. 2009, Bommarius et al. 2010, Pei et al. 2014).

Gene cloning and expression (recombinant DNA technology), is a process by which genes can exert their function (.i.e. convert the information stored in a gene to a functional product) and is the only way of isolating long genes or those that have never been studied before (Brown 2006). *E. coli* is one of the organisms of choice for the production of recombinant proteins and its use as a cell factory is well-established (Samuelson 2011). It has become the most popular expression platform with many molecular tools and protocols at hand for high-level production of heterologous proteins due to its rapid growth, a variety of portable vectors, relatively simple genetics, and the

potential for high-density cultivation (Samuelson 2011, Rosano and Ceccarelli 2014). However, difficulties have been encountered in the expression of antimicrobial proteins/peptides in bacterial and other hosts due to their toxicity to host cells, susceptibility to proteolysis and low expression levels (Doherty et al. 1993, Dong et al. 1995, Jin et al. 2006, Chen et al. 2008). As a result, various expression systems with AMPs fused to a partner protein have become popular as the most effective method of recombinant AMP production because they have reduced toxicity, enhanced product stability and recovery (Wei et al. 2005, Moon et al. 2006, Xu et al. 2006, Chen et al. 2009). However, although these fusion technologies overcome some of the disadvantages of traditional recombinant methods mentioned above, some of these methods are still being studied and many of them are still commercially unavailable.

In Chapter three, 5 putative antimicrobial peptide genes (Gene 6, 17A, 17B, *palG*1 and *palG*2) were discovered through functional and sequence based screening of the fosmid metagenome library. In addition, six genes (H-G1 to H-G6) were identified in an already existing cow rumen metagenomic dataset (Hess et al. 2011). This chapter therefore, describes the amplification, expression and purification of these never before studied potential antimicrobial genes (predicted AMPs > 22 AAs) using a conventional and *E. coli* expression system in the first instance, in order to enable further characterisation of the antimicrobial potential of the genes identified. AMPs ≤22 AAs in length were chemically synthesised (section 2.7) before characterisation of their activity.

4.2 Materials and methods

4.2.1 Amplification of antimicrobial genes

Fosmid DNA to be used as templates for PCR amplification were extracted as described in section 2.6.2. For the amplification of H-G 1 to 6, DNA was extracted from milled

rumen samples from our laboratory, and not from the original rumen sample from which the data was generated (see section 2.6.2.1). Primers used for the amplification of the antimicrobial genes were designed using the online tool available at http://tmcalculator.neb.com/#!/. As none of the genes were predicted to have a signal peptide (using the SignalP 4.1 server), the primers were designed to start and stop at the first predicted methionine and at the last stop codon respectively in order to conserve the reading frame and take account of the entire gene of interest, with a Tm^{o} greater than $50^{o}C$. All designed primers were synthesised by Sigma Aldrich, Dorset, UK. It was not necessary to add restriction enzymes or a Histidine tag (His-Tag) sequence to the primer sequences of genes to be expressed using the pTrcHis TOPO® TA Expression kit as the pTrcHis-TOPO® cloning vector carries a His-Tag and allows for the ligation reaction between the amplified gene insert and the vector without requiring an enzyme restriction step. The primers used are presented in Table 4.1 below.

Table 4.1 Primers for amplification of antimicrobial genes in putative positive fosmid clones using the pTrcHis TOPO® TA Expression System (F, forward primer and R, reverse primer).

Sample ID/ORF (Template)	Antimicrobial gene amplified		Primer sequence (5'-3')	Expected Product size (bp)
SABPL5 C17/11		*F*	ATGATTACTCTGGTGCGTGAGG	
	Gene 6	*R*	CTATTCACATATGGTGTAAATATACCGC TGTCG	555
SAB PL12(1) C3/9		*F*	ATGGATAAACAGAAGGTTATCATATCG CTCC	
	Gene 17A	*R*	TTAGTTCCCCTCGCGATAAAAGTC	1053
SAB PL12(1) C3/50		*F*	ATGGGCTGTTCTATCGAGTGG	
	Gene 17B	*R*	CTACGACGAGATGCTTGCACGATTG	243
SAB PL27 L10/66		*F*	ATGAGGCTGTCACACGTTTG	
	palG1	*R*	TCACCAATCTGTATGGCACCG	216
SAB PL27 L10/73		*F*	ATGAGCGCCATGAGGCAC	
	palG2	*R*	TCAGACAAGGCATATTTCAAGGATGCC	207

Table 4.2 Primers for amplification of antimicrobial genes from metagenomics sequence dataset (F, forward primer and R, reverse primer).

Antimicrobial gene amplified		Primer sequence (5'-3')	Expected product size (bp)
H-G1	*F*	CACCATGGGATTTCGTGAATCA	213
	R	CACTTTCTTCGGCTTCTTTGTCG	
H-G2	*F*	CACCCGGGGCCTTTTTG	444
	R	AGCATCAACATGCTGGAAGCC	
H-G3	*F*	CACCCTTCTCTTTCTTGCTCTTTCT	156
	R	GCTGAAAAGAAGCAAGCAGAAAA	
H-G4	*F*	CACCATGGACCATCCCTGC	1971
	R	AGATAAAATCAACCCCACCATCG	
H-G5	*F*	CACCATGAGTGATGTAATTGACGATAA	788
	R	ATGATATCGTTAAATATCTTTACGCTTCTT	
H-G6	*F*	CACCATGCTATCATTCCTATTCATGC	138
	R	AAGAAACTTCCTCTTCAGGAATCTCTT	

All primers were diluted to a concentration of 100 µM and a total volume of 50 µl PCR reaction was set up as follows: 2 µl DNA template, 1 µl each of forward and reverse primers, 39.5 µl molecular grade water and 1 µl Titanium® *Taq* DNA Polymerase (Clonetech- Takara Bio Europe/SAS, France). Taq was initially activated for 1 minute at 95^{o}C, followed by 30 cycles of 95^{o}C for 30 seconds, 68^{o}C for 1.5 minutes, followed by a final extension step at 68^{o}C for 1.5 minutes. PCR products were verified by electrophoresis on a 1.5% agarose gel using a 1kb ladder. Gel image was taken after exposure to UV using the Gel Doc^{TM} XR^{+} system (BIO-RAD Hertfordshire, UK). Subsequently, the band of interest was excised with a clean sterile scalpel under a Dark Reader blue transilluminator (Clare Chemical Research Inc. USA) and DNA was purified and eluted as described in section 2.6.5.

4.2.2 *In vivo* cloning of antimicrobial genes

4.2.2.1 Cloning using the pTrcHis TOPO® TA Expression System

The cloning of antimicrobial genes was initially carried out using the pTrcHis TOPO® TA Expression kit. Briefly, the cloning reaction was set up by mixing 4 µl of purified PCR amplified gene (section 4.2.1) with 1 µl of pTrcHis TOPO® cloning vector. The reaction was gently mixed and incubated for 5 minutes at room temperature. Thereafter, 2 µl of the cloning reaction was added to a vial of OneShot® *E. coli* TOP10 cells and left to incubate on ice for 30 minutes, after which, the cells were heat shocked for 1 minute at 42^{o}C and then immediately placed on ice. Subsequently, the cells were added to 250 µl room temperature SOC medium (Super Optimal broth (SOB) with catabolite repression i.e., with 20 mM glucose) and incubated with horizontal shaking at 37^{o}C for 60 minutes. Volumes of 30 µl and 70 µl were then plated onto pre-warmed LB plates supplemented with 100 µg/ml ampicillin and 0.5% glucose and incubated overnight at 37^{o}C.

4.2.2.2 Confirmation of cloning reaction

Sequencing and tip-dip PCR were used to analyze positive transformants. Five colonies from each transformation were analyzed for correct size sequence and orientation. The selected colonies were cultured overnight in LB medium containing 100 µg/ml ampicillin and 0.5% glucose. Their plasmid DNA was then isolated as described in section 2.6.2. The Xpress™ Forward sequencing primer for pTrcHis-TOPO® (5´-TATGGCTAGCATGACTGGT-3´) was then used to sequence the insert by Sanger sequencing (reaction was made up of at least 250 ng and 1.6 ρM of plasmid DNA and primer respectively). Sequence alignments confirming that transformants had the correct sequence can be found in the file ‘cloning transformants alignment’ in CD/Figshare.

For analysis of positive transformants by tip-dip PCR, 1 ml aliquots of the same selected five colonies were cultured overnight and lysed by heating for 10 minutes at 95^{o}C in sterile 1.5 ml microcentrifuge tubes. The cell debris was then pelleted by centrifugation at 13,000 x g for 2 minutes. The supernatant was used as the template for PCR. The PCR reaction was set up in a total volume of 50 µl as follows: 2 µl of template DNA, 1 µl each of gene specific forward primer (Table 4.1) and vector specific reverse primer (5´-GATTTAATCTGTATCAGG-3´), 21 µl molecular grade water and 25 µl MyTaq™ Red Mix (Bioline, UK Ltd, London UK). Initial Taq activation was performed at 95^{o}C for 1 minute, followed by 35 cycles of 95^{o}C for 15 seconds, at insert specific annealing temperature for 15 seconds and an extension step at 72^{o}C for 10 seconds, then a final extension step at 72^{o}C for 7 minutes and holding at 4^{o}C. Again, PCR products were verified by electrophoresis on a 1.5% agarose gel using a 1kb ladder. A positive PCR control was also prepared using the control PCR template (expected size of 750bp) and primers provided with the pTrcHis-TOPO® Expression kit.

After analysis and confirmation, single colonies of positive transformants with correct inserts were grown at 37^{o}C with shaking in LB broth containing 100 µg/ml ampicillin and 0.5% glucose until culture reached mid-log (OD_{600}nm of ~ 0.5-0.7). Aliquots (2 ml) were then stored in 30% (v/v) sterile glycerol in cryovials at -80^{o}C.

4.2.3 Expression of His-tagged antimicrobial proteins

Firstly, a pilot expression was carried out to determine the best conditions for maximum expression of each protein. A single recombinant *E. coli* colony of the clones chosen for each protein was inoculated into 2 ml of LB broth containing 100 µg/ml ampicillin and grown overnight at 37°C with shaking (225-250 rpm). The following day, 10 ml of LB containing 100 µg/ml ampicillin was inoculated with 0.2 ml of the overnight culture and

incubated at 37°C with vigorous shaking to an OD_{600}nm = 0.6 (mid-log phase), after which 1 ml aliquot of cells was removed, centrifuged at maximum speed (15,000 x g) in a microcentrifuge for 30 seconds, and the cell pellet was frozen at -20°C after aspirating the supernatant. This was taken as the zero time point. IPTG was then added to a final concentration of 1 mM (9 µl of a 1 M IPTG stock to 9 ml) and grown at 37°C with shaking. Every hour for five hours, 1 ml samples were removed and treated in the same way as above after normalising cell densities. Pellets from all time points were subsequently analyzed by sodium dodecyl sulphate polyacrylamide gel ((SDS-PAGE)-Section 4.2.4) to determine the optimal time point for maximum expression. TOP10 cells containing pTrcHis-TOPO®/lacZ and TOP10 cells that did not contain any vector were used as positive and negative expression controls respectively. Expression of proteins was then scaled up to 1 litre volumes. Cells were harvested by centrifugation (3,000 x g for 10 minutes at 4^oC) at the pre-determined optimal time point. Pellets were then stored at -80^oC for subsequent protein purification (section 4.2.5)

4.2.4 Polyacrylamide gel electrophoresis

Proteins were analysed using SDS-PAGE on a 20% resolving gel (1.5 M Tris-HCl (pH 8.8), 0.4 % SDS, 20% (w/v) acrylamide) and a 5 % stacking gel (0.5 M Tris-HCl (pH 6.8), 0.4 % SDS, 5% (w/v) acrylamide), cross linked by addition of 40 % N, N‟-bis acrylamide solution (BIO-RAD Hertfordshire, UK)) (Laemmli, 1970). Polymerisation of both the resolving and stacking gels was initiated by addition of 0.5% freshly made 10% (w/v) ammonium persulphate (APS) and 0.4 and 0.1% (w/v) *N,N,N‟,N‟*-Tetramethylenediamine (TEMED) respectively. The running gel solution was poured between the glass plates with a pipette, with about 1/4 of space free for the stacking gel. It was then carefully covered with ultra pure water. The resolving gel was then left to polymerize for ~30 minutes (a clear line appears between the gel surface and the water

on top when polymerization is complete). The water is then discarded and the stacking gel solution (prepared as described above) is carefully poured into the glass plates with a pipette to avoid formation of bubbles. This was followed by the insertion of appropriate sized combs, after which the stacking gel was allowed to polymerize for at least 60 minutes. Then, the combs were carefully removed and the gels were transferred into an electrophoresis tank with bottom and top reservoirs, filled with fresh 1X Tris-glycine-SDS Buffer. Protein samples and protein molecular weight markers (precision plus (BIO-RAD Hertfordshire, UK) or NEB #P7703 (New England Biolabs Hitchin, UK)) were then loaded onto the gels. Vertical electrophoresis was run at 25 mA (for two minigels 100 x 80 x 1mm) until the dye front (bromophenol blue line) reached the base of the gel.

Preparation of protein samples was as follows: when the cell pellets from the different expression time points were collected and prepared as described in Section 4.2.3, they were each resuspended in 100 µl of 1X SDS-PAGE sample buffer (17.5% double distilled water, 0.5 M Tris pH 6.8, 5 ml 25% Glycerol, 10% SDS, 2.5% 2-β-mercaptoethanol and 0.1% bromophenol blue). They were then heated at 95^{o}C for 5 minutes and centrifuged briefly, after which 5 µl of each sample was analysed on an SDS-PAGE gel.

Upon completion of electrophoresis, protein bands were detected by first fixing the gels with a solution of 40% (v/v) methanol and 10% (v/v) glacial acetic acid for at least 40 minutes, before staining the gel with Coomassie stain (0.25% Coomassie Brilliant Blue R-250 (Sigma Aldrich, UK), 50% (v/v) methanol and 10% (v/v) glacial acetic acid for up to 2 hours. Background stain was then removed by a destaining solution (40% (v/v) methanol and 10% (v/v) glacial acetic acid). Gel images were taken using the GS-800^{TM} calibrated densitometer (BIO-RAD Hertfordshire, UK). The recombinant proteins were

identified by a band of increasing intensity in the expected size range with the His-tag contributing ~ 3 to 4 kDa to expressed protein. The negative control (*E. coli* TOP10 only cells) were used to distinguish recombinant proteins from background proteins while the positive control (pTrcHis-TOPO®/lacZ which should yield a 40 kDa protein between 3- 4 hours) was used to confirm that growth and induction was done successfully.

4.2.5 Purification of His-tagged proteins

Purification of the proteins was carried out under native conditions using the Amicon® Pro Purification System (Merck Millipore Ltd Carrigtwohill, Ireland).

4.2.5.1 Preparation of *E. coli* lysate for purification under native conditions

Briefly, the cell pellets from a scaled up expression (1000 ml culture) of recombinant antimicrobial proteins prepared as described in section 4.2.3 were resuspended in 40 ml native binding (lysis) buffer (15 mM imidazole, 500 mM sodium chloride (NaCl), 20 mM Tris-hydrochloride pH 7.9). Lysozyme from chicken egg white (Sigma-Aldrich, UK) to a final concentration of 1 mg/ml and four tablets of protease inhibitor cocktail (one tablet per 10 ml) (Roche, Burgess Hill, UK) were then added, mixed and incubated at room temperature for 30 minutes. The solution was then sonicated using a Vibracell VCX130 (Sonics and Materials, USA) on ice (twelve 10-second bursts at 50% amplitude with a 30-second cooling period between each burst). Afterwards, the lysate was centrifuged at 7,000 x g for 15 minutes to pellet the cell debris and the supernatant (lysate) was transferred to a fresh tube and chilled at -20^{o}C until purification.

4.2.5.2 Resin preparation in the Amicon® Pro purification system

Up to 1000 µl of previously vortexed packed Nickel-nitrilotriacetic acid (Ni-NTA) Agarose slurry (0.5 ml bed volume) (Qiagen, Crawley, UK) was pipetted into the

Amicon® Pro exchange device and centrifuged in a swinging-bucket rotor at 1,000 x g for 1 minute to remove the storage buffer from the resin. The binding buffer (5 ml) was then added to the resin and centrifuged at 1,000 x g for 1 minute.

4.2.5.3 Protein binding and wash in the Amicon® Pro purification system

Approximately 9 ml of lysate (from 4.2.5.1) was added to the equilibrated resin matrix and incubated at room temperature for 60 minutes with gentle agitation (shaking 200 rpm) on a rotary shaker. The device was thereafter centrifuged at 2,000 x g for 4 minutes and sample flow-through was recovered. Three wash steps were performed by adding 9 ml wash buffer (35 mM imidazole, 1 mM NaCl, 20 mM Tris-HCl pH 7.9) and centrifuging at 2,000 x g for 4 minutes. Wash fractions were recovered following each wash step.

4.2.5.4 Sample elution, buffer exchange and concentration in the Amicon® Pro purification system

For simultaneous elution and concentration of purified proteins, the Amicon® Ultra-0.5 (molecular weight cut-off 3 kDa) was inserted into the exchange device and placed back into the device holder. The resin was then gently resuspended in up to 1.5 ml of elution buffer (400 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl pH 7.9) and centrifuged at 4,000 x g for 60 minutes. A total volume of 1.5 ml of the desired exchange buffer (100 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (pH 7.5)) was then added to the exchange device/Amicon® Ultra-0.5 assembly and centrifuged at 4,000 x g for a further 60 minutes. The purified protein samples were subsequently collected from the Amicon® Ultra-0.5 device by reverse spin. A 2 ml collection tube was placed over the top of the Amicon® Ultra-0.5 device held in an upright position. The assembly was then inverted and centrifuged in a microcentrifuge with fixed-angle rotor at 15,000 x g for 60 minutes. Sample protein concentration was determined at an

absorbance of 280 nm using the BioTek's Epoch™ Multi-Volume Spectrophotometer (BioTek Instruments, Inc. Vermont, USA). The concentration of the protein was calculated as the ratio of A280nm to the extinction coefficient absorbance (Abs 0.1% (=1 g/l) obtained inputing protein sequence into the ExPASy ProtParam tool available at http://web.expasy.org/cgi-bin/protparam/protparam) (Gasteiger et al. 2005).

4.2.6 *In vitro* expression of antimicrobial genes

In vitro expression of antimicrobial genes was performed for those genes which were difficult to express *in vivo* in *E. coli* cells. This was completed using the PURExpress® *In Vitro* Protein Synthesis kit (New England BioLabs Inc, Hitchin, UK). Primers for *in vitro* expression were designed as described in the manufacture's manual. Adaptor sequences (homologous to part of the regulatory region DNA) were added to the 5´ and 3´ ends of primers of the genes of interest as shown in Figure 4.1 below.

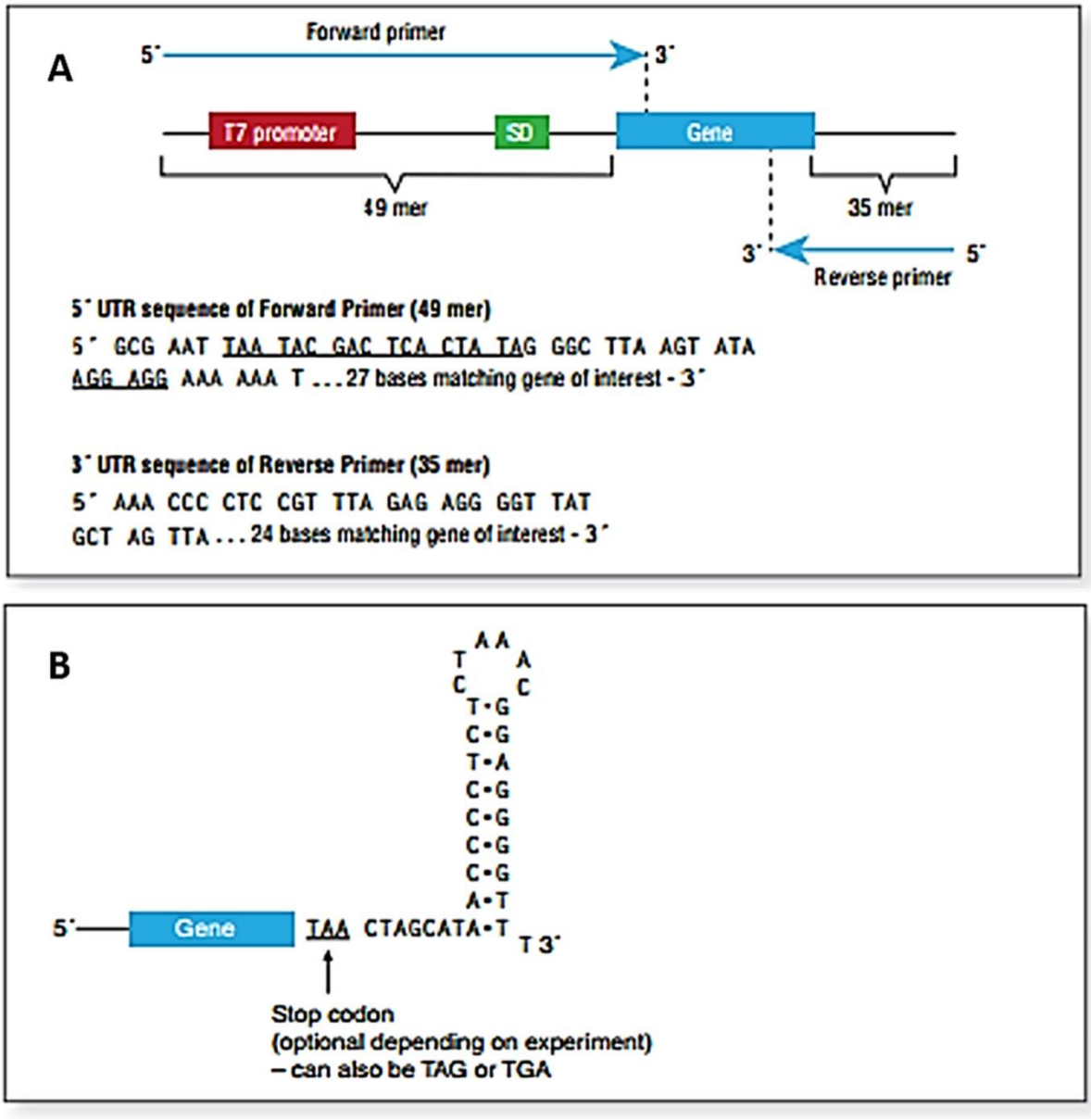


Figure 4.1 Suggested primer design for PCR for generation of template DNA for use with PURExpress (A), suggested primer design sequence for PCR. (B), Illustration of 3´ UTR Stem Loop of PCR Templates for PURExpress.

The primers used for amplification and preparation of template DNA of antimicrobial genes for use with PURExpress *in vitro* expression are given in Table 4.3 below. The expression reaction was assembled on ice in microcentrifuge tubes in a total volume of 25 µl as follows: 10 µl Solution A, 7.5 µl Solution B, 1 µl (20 units) of Murine RNAse inhibitor (NEB #M0314, Herts, UK), at least 500 ng DNA template (amplified as described in section 4.2.1), and nuclease free water.

Table 4.3 Primers for amplification of antimicrobial genes for use with PURExpress (F, forward primer and R, reverse primer).

Gene name	Primer sequence (5'-3')
Gene 6 -*F*	GCGAATTAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATA TGATTACTCTGGTGCGTGAGGATATTTGG
Gene 6-*R*	AAACCCCTCCGTTTAGAGAGGGGTTATGCTAGTTA CTATTCACATATGGTGTAAATATACC
Gene 17A - *F*	GCGAATTAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATA TGGATAAACAGAAGGTTATCATATCGCTCCAG
Gene 17A -*R*	AAACCCCTCCGTTTAGAGAGGGGTTATGCTAGTTATTAGTTCCCCTCGCGA TAAAAGTC
Gene 17B -*F*	GCGAATTAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATA TGGGCTGTTCTATCGAGTGGATGTTG
Gene 17B -*R*	AAACCCCTCCGTTTAGAGAGGGGTTATGCTAGTTACTACGACGAGATGCT TGCACGATTG
*palG*1 -*F*	GCGAATTAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATA TGAGGCTGTCACACGTTTGTAGTCAGAG
*palG*1 -*R*	AAACCCCTCCGTTTAGAGAGGGGTTATGCTAGTTATCACCAATCTGTATGG CACCGACC
palG2 -*F*	GCGAATTAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATA TGAGCGCCATGAGGCACGATGTACTTCC
PalG2 -R	AAACCCCTCCGTTTAGAGAGGGGTTATGCTAGTTATCAGACAAGGCATAT TTCAAGGATGCC

The reaction was then incubated at 37^{o}C for 4 hours. Dihydrofolate reductase (DHFR) positive control template was used to determine a successful expression reaction. Upon completion of *in vitro* expression, 2.5 µl of expression product was added to 10 µl of 4X SDS loading buffer, heated at 100^{o}C for 5 minutes and analysed by SDS-PAGE as described in section 4.2.4.

Purification of synthesized proteins was carried out using reverse His-tag purification as described in the user manual. Briefly, 10 mM magnesium acetate was added to the reaction to a final volume of 125 µl in order to increase the volume and make handling of the sample easier. The diluted reaction mixture was then applied to an Amicon Ultracel 0.5 ml-100K spin concentrator (0.5 ml maximum load volume) (Merck

Millipore Ltd Carrigtwohill, Ireland) and centrifuged for 30–60 minutes at 15,000 x g at 4°C. The permeate/flow-through was thereafter transferred to a new 2 ml round-bottom microcentrifuge tube with a leak-proof cap. This was followed by the addition of 0.25 volumes Ni-NTA Agarose and mixed thoroughly at 4°C for 60 minutes to allow His-tagged components to bind to the resin. The reaction mixture slurry was subsequently transferred to an empty Bio-Rad micro-spin column and centrifuged for 60 minutes at 15,000 x g at 4°C. The eluate containing purified protein was collected and analysed by SDS-PAGE for presence of purified protein.

4.3 Results

4.3.1 Cloning expression and purification in *in vivo* expression systems

PCR amplification of antimicrobial genes was successful for all the genes as illustrated on Figure 4.2 and 4.3. Bands of the correct size were excised from the gel before proceeding to cloning to ensure subsequent clones were carrying the correct PCR product as some had more than one amplicon present. Five colonies per transformation for each gene cloned into the pTrcHis-TOPO® vector were analysed for size and orientation of the insert. The electrophoresis results for tip-dip PCR presented in Figure 4.4 show that chosen clones were confirmed as carrying the genes of the correct size. Figure 4.4 also shows the clones that were stored as glycerol stocks and chosen for protein expression. The antimicrobial proteins were expressed with an N-terminus six-histidine tag in *E. coli* in order to enable purification and investigation of their biochemical properties. The pilot protein expression assay showed that protein expression was optimal 4 hours after induction of the clone cultures with 1 mM IPTG, as illustrated by SDS-PAGE results in Figure 4.5. This Figure also shows the appearance of target protein bands visualised on Coomassie-stained gels compared to the positive and negative expression controls at different expression time points after

induction with IPTG. Based on the level of expression of the proteins in the pTrcHis-TOPO® TA expression system (Figure 4.5), only proteins *palG*1 was chosen for purification, as there was no visible increase in band intensity over time during expression of the other proteins (Gene 6, 17A, 17B and *palG*2). This may be because the proteins were insoluble as they could not be detected on SDS-PAGE (Figure 4.5 A, B, C, E) or because high expression levels of the proteins induced by IPTG was toxic to the host *E. coli* cells. Cultures of *palG*1 transformants were scaled up appropriately for harvesting cell pellets for protein purification. The recombinant proteins were purified in native conditions in order to preserve their activity as described in Section 4.2.5. Figure 4.6 shows the SDS-PAGE analysis of the purification fractions from *palG*1. The purification protocol routinely yielded 0.4 to 0.8 mg/ml of purified protein from 1000 ml cultures.

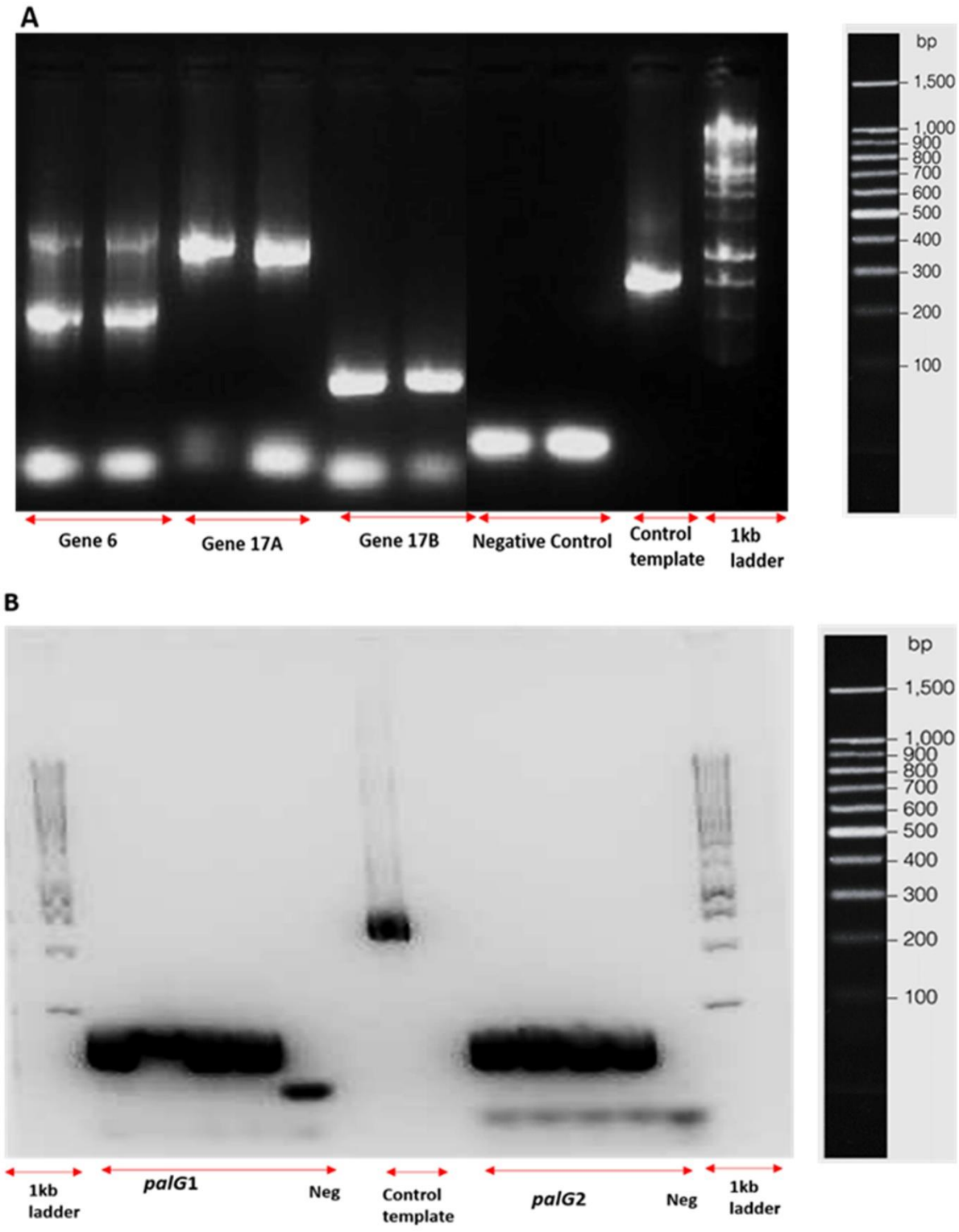


Figure 4.2 PCR amplification product analysis on 1.5% agarose gel of antimicrobial genes- using primers in Table 4.1. **Neg**, negative control. Expected size is 555, 1053, 243, 216 and 207bp for Gene 6, 17A, 17B, *palG*1 and *palG*2 respetively.

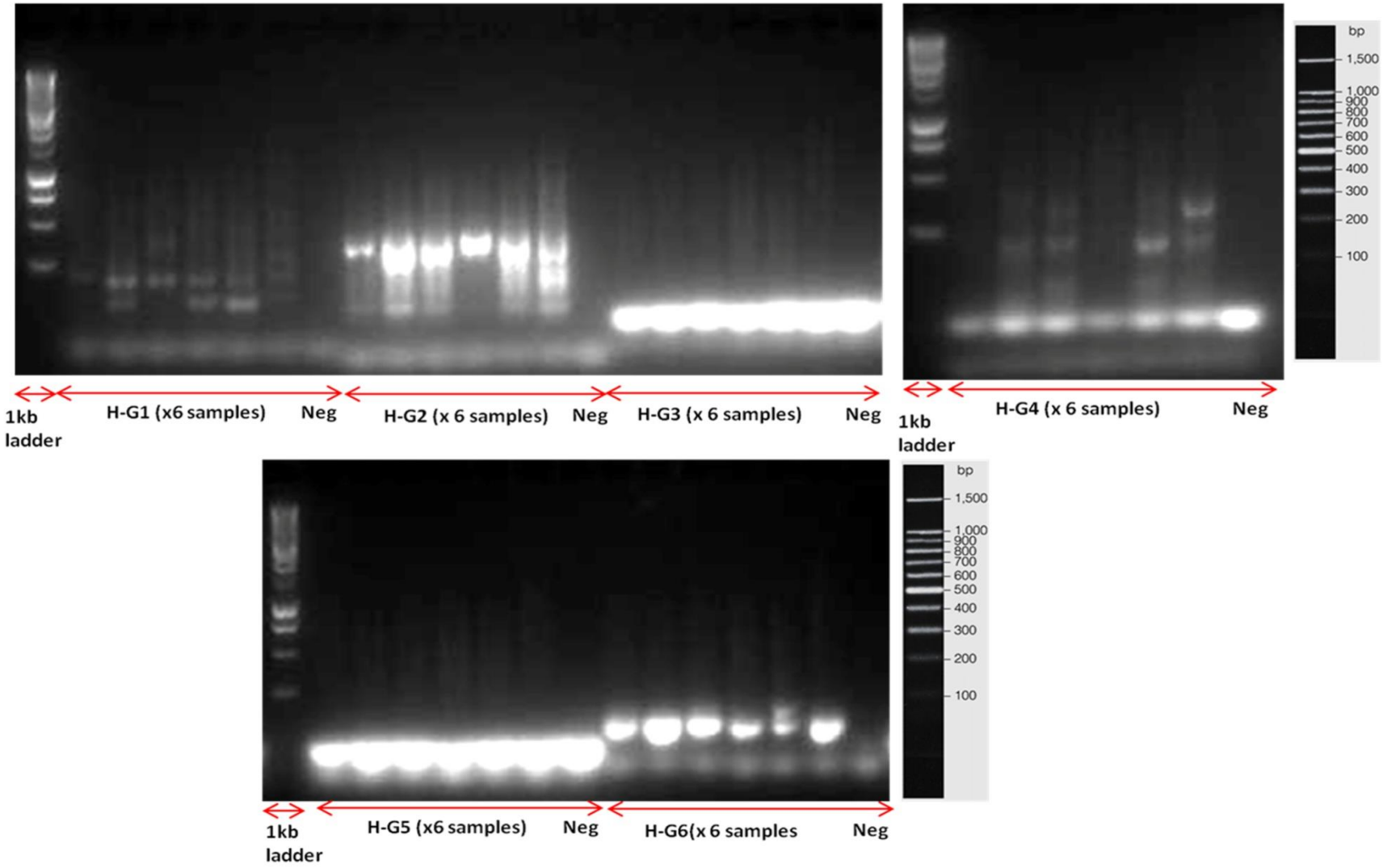


Figure 4.3 PCR amplification product analysis on 1.5% agarose gel of antimicrobial genes from Hess etal. (2011) dataset- using primers in Table 4.2. Each gene was amplified using 6 rumen samples, **Neg**, negative control. Expected size is 213, 444, 156, 1971, 788 and 1386bp for H-G1 to H-G6 respetively.

The antimicrobial genes from the IBERS fosmid library were successfully amplified by PCR using fosmid DNA from the clones from which they were identified (Figure 4.2 above). Only H-G2 and H-G6 had specific PCR amplified product band for at least one rumen sample used. H-G1 and H-G4 had unspecific DNA products while H-G3 and H-G5 were not amplified at all.

Also, as can be seen in Figure 4.4 below, Tip-dip PCR analysis confirmed that cloned PCR products were of the correct size.

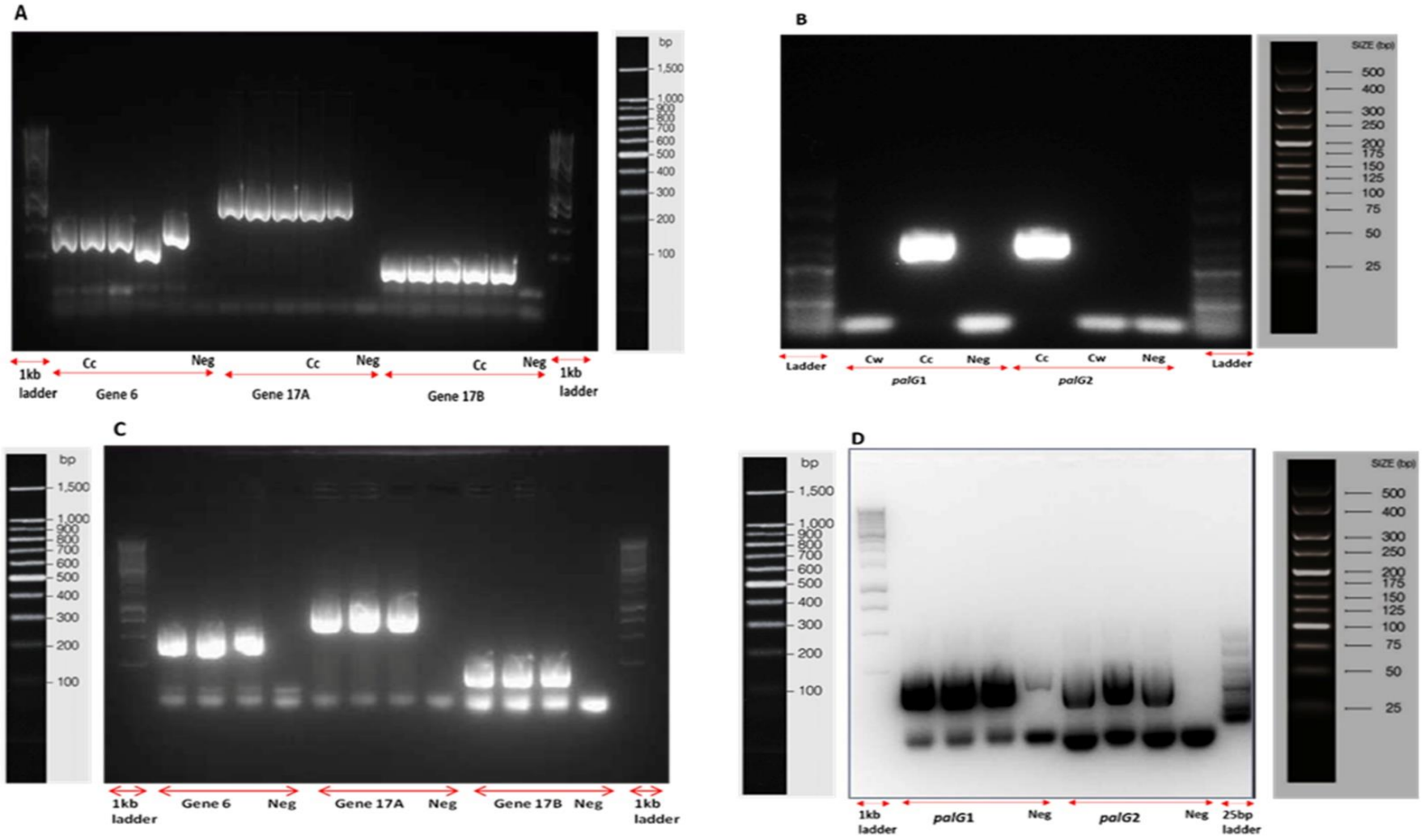


Figure 4.4 Tip-dip PCR amplification product analysis on 1.5% agarose gel of transformants (colonies) of antimicrobial genes using gene specific forward primers and pTrcHis-TOPO® reverse primers. **Neg**, negative control, **Cc**, clones with correct insert size (one of which was chosen for protein expression), **Cw**, clone with no amplified insert. Expected size is 555, 1053, 243, 216 and 207bp for Gene 6, 17A, 17B, *palG*1 and *palG*2 respetively.

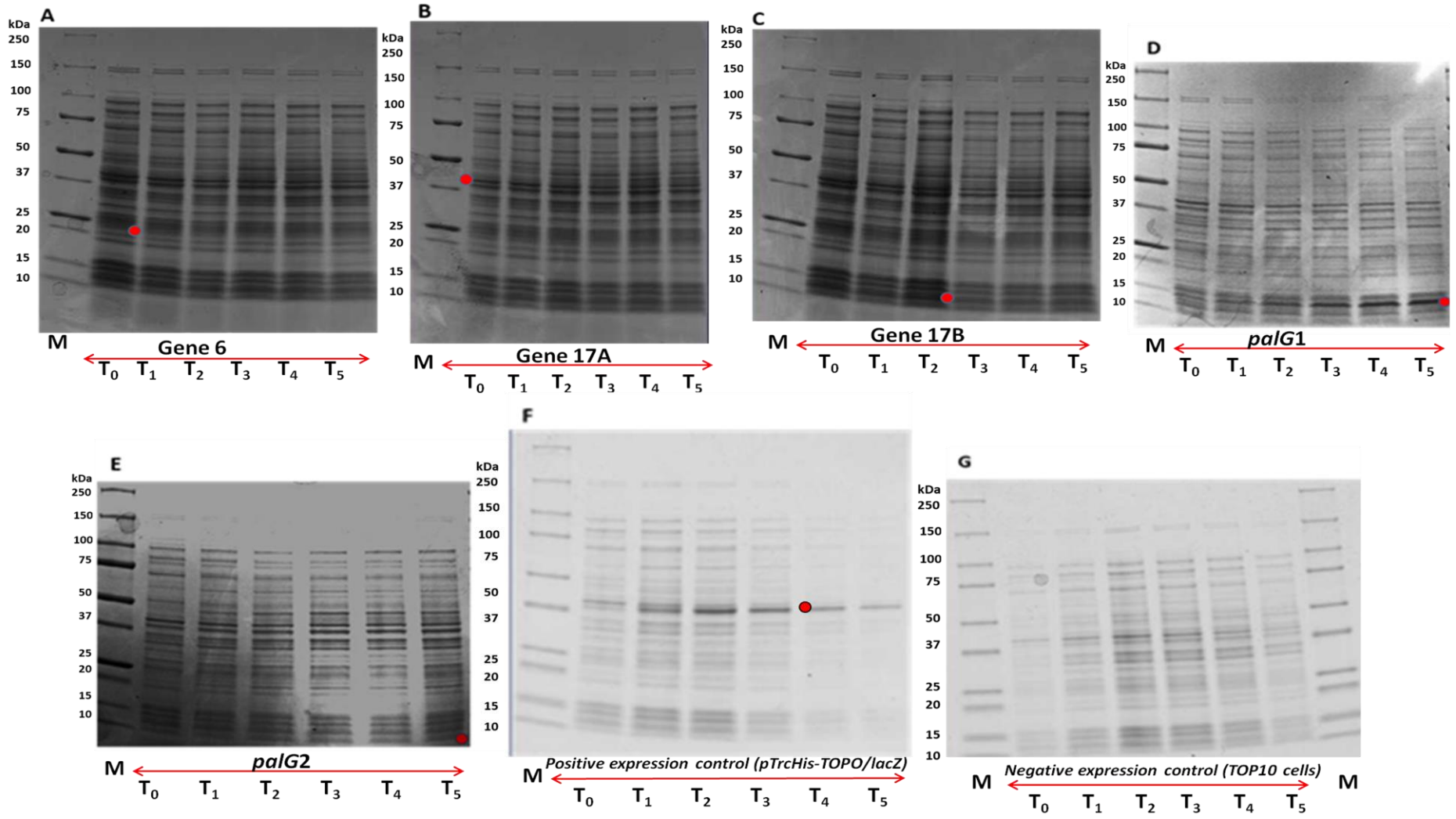


Figure 4.5 SDS-PAGE analysis of expression of the His-tagged antimicrobial proteins in *E. coli* TOP10 cells - from 0 to 5 hours after induction with 1 mM IPTG. Pellets from 1 ml samples taken before induction (**T_0**) and hourly after induction (**T_1, T_2, T_3, T_4 and T_5**). **M** is the Protein Ladder. The red dot indicates the expected size of protein of interest. Expected sample size for proteins Gene 6, Gene 17A, Gene 17B, *palG*1, *palG*2, and pTrcHis-TOPO/LacZ are 21.4, 38.71, 9.98, 8.35, 7.32 and 40 kDa (±3-4 kDa from His-tag) respectively.

Only *palG*1 was successfully expressed over time after induction with IPTG. Negative and positive control proteins were as expected.

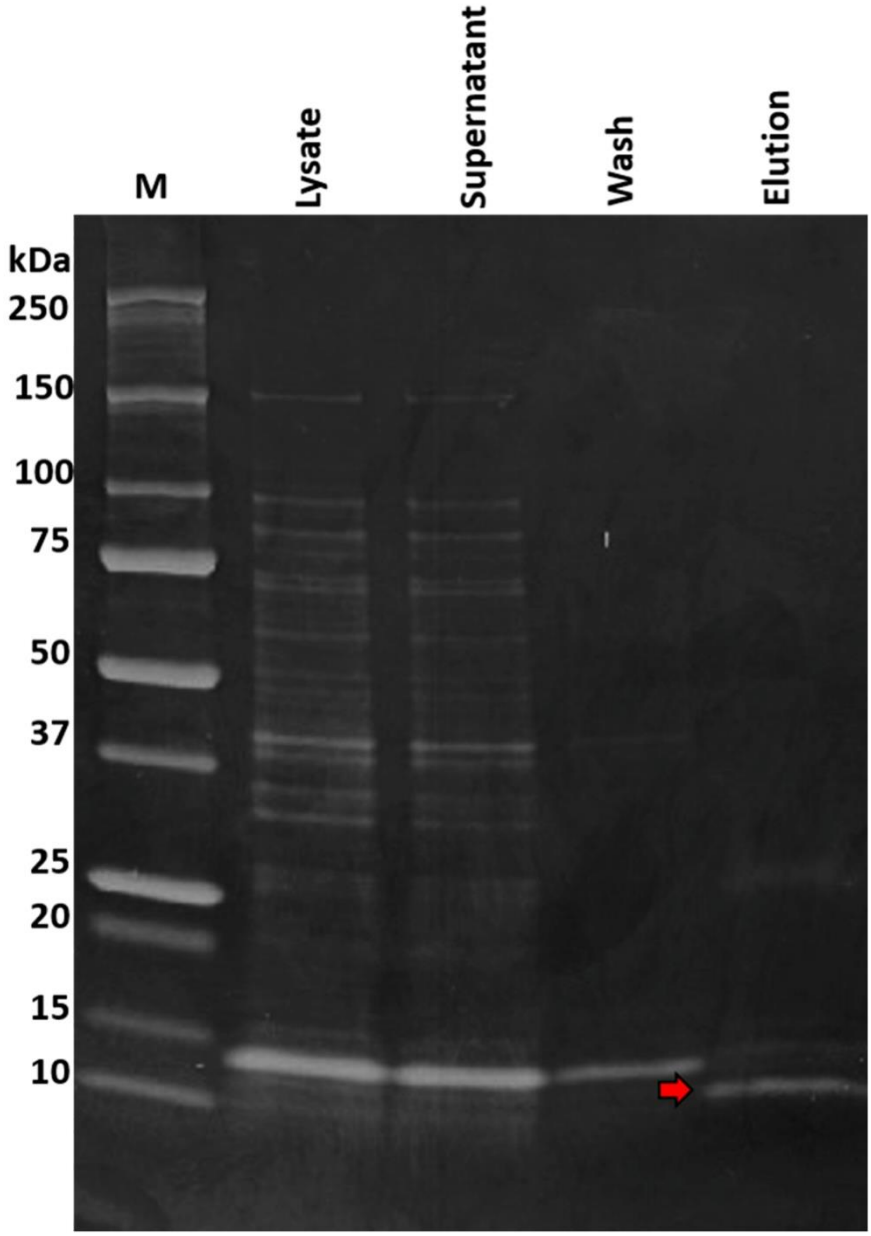


Figure 4.6 SDS-PAGE analysis of purification steps of *palG*1 protein expressed in *E. coli* TOP10 cells on a 20% denaturing polyacrylamide gel (5 hours after induction with 1 mM IPTG). The red arrow indicates band of purified protein of interest. Expected size is 8.35 (±3-4 kDa from His-tag).

Pure protein was obtained after purification as can be seen in Figure 4.6 above.

4.3.1 Cloning expression and purification in *in vitro* expression systems

Genes 6, 17A, and 17B were successfully expressed *in vitro* using PURExpress (*E. coli* lysate with a T7 promoter) as shown in Figure 4.7 below. However, several attempts to purify proteins expressed in this *in vitro* system were unsuccessful (Figure 4.8 in CD/Figshare).

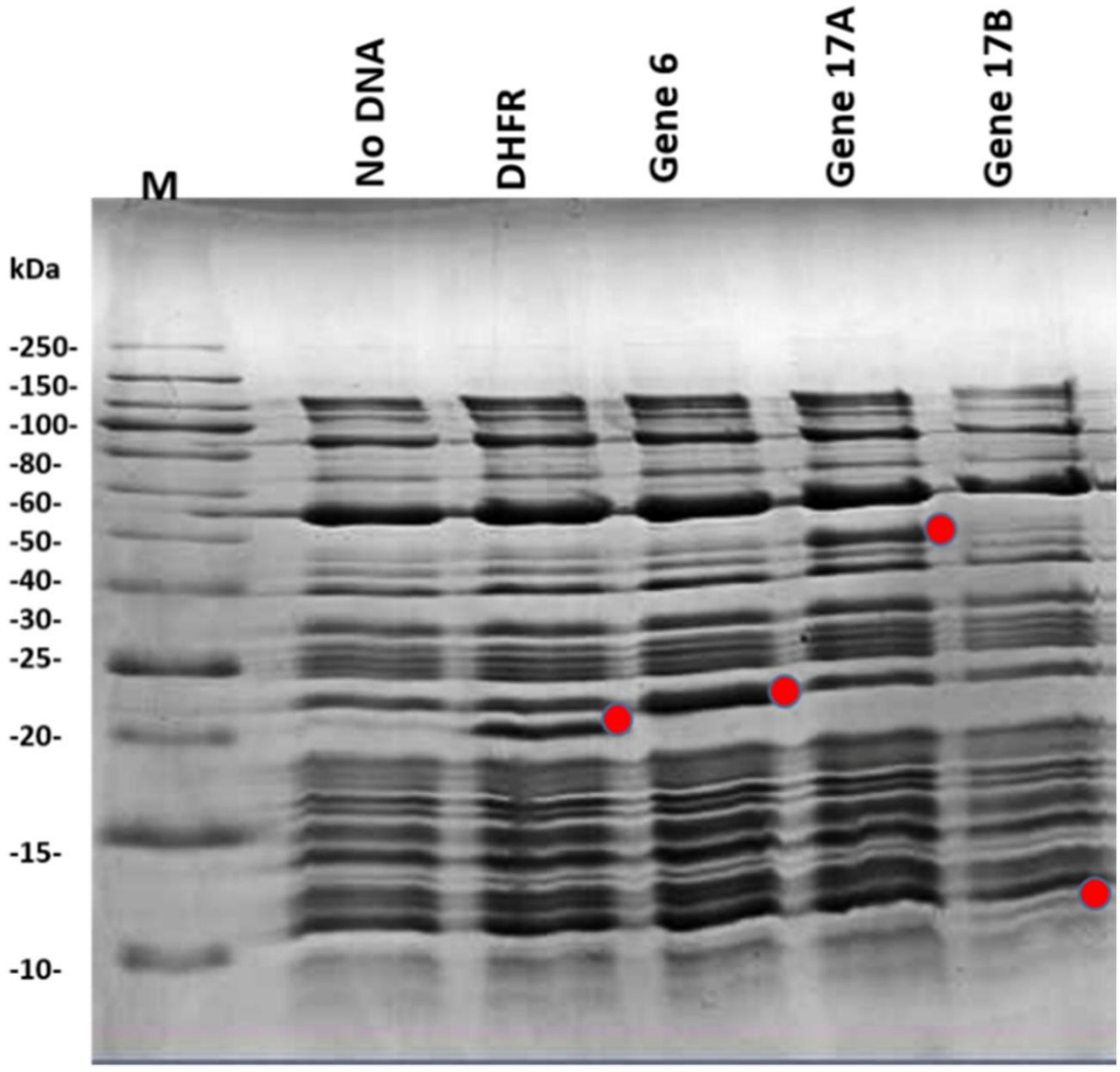


Figure 4.7 SDS-PAGE analysis of PURExpress reaction for antimicrobial proteins after 4 hours incubation. The red dots indicate the protein band of interest. **M**, Protein Ladder. **DHFR**, control DNA template. Expected sample size is 23, 21.4, 38.71, 9.98 kDa for DHFR, Gene 6, Gene 17A, Gene 17B respectively.

4.4 Discussion

Although, PCR amplification was successful for all antimicrobial genes identified from the in house rumen metagenomic library, *in vivo* expression of selected putative antimicrobial genes (cloned into pTrcHis TOPO vector and transformed into chemically competent *E. coli* TOP10 cells) was only successful for *palG*1 (Figures 4.5 and 4.6). No visible expression profile was observed over time for all other proteins even when expression conditions were changed. Positive control showed an increasing expression over time and as expected, negative control had no expression profile.

DNA amplification of Hess et al, (2011) genes (H-G1 to H-G6) was problematic. H-G2 and H-G6 were the only AMPs in this group successfully amplified without unspecific DNA amplicons in at least one rumen DNA template/sample used. An attempt to recover these DNA amplicons through gel purification yielded DNA products very low in concentration and sequencing showed that the gene was only partially amplified (results not shown). This could be due to the fact that many of the genes were partial genes and some had unknown bases and/or no start codons. Another reason could be that the genes were being amplified from different rumen samples and not the original sample used to generate the data. Due to time constraint, attempt to express the H-G1 to H-G6 genes was aborted. It will be necessary to redesign primers and attempt to amplify these genes again in the future and attempt to successfully express them. In the meantime, H-G2 and H-G4, which were both 22 AAs long were chemically synthesized to determine their antimicrobial activity (discussed in Chapter Five).

All in-house proteins were successfully expressed in an *in vitro* expression system. However, several attempts to purify these genes were unsuccessful. This may be due either to low expression levels or the reverse purification efficiency. Proteins of interest may have expressed well but not in high yield, and even with high protein yield, it may

still be difficult to go through the purification if the protein was insoluble. Expression of *palG*2 was still unsuccessful even in the *in vitro* system (results not shown).

The preparation of the cell pellet for purification is a critical step for protein yields, as the lysis should be as efficient as possible. A yield of around 0.2 to 0.5 mg/ml of purified *palG*1 protein was routinely achieved from 1 litre cultures. This yield is not as efficient as those reported in previous studies where purified antimicrobial proteins ranging in concentrations of 0.5 mg/ml (Guerreiro et al. 2008) to 2.5 mg/ml (Pei et al. 2014), 0.8 mg/ml (Zorko et al. 2009), 10 mg/ml and up 100 mg/ml (Lee et al. 2008) were retrieved respectively from 1 litre culture using Ni-NTA columns. It should be noted that different prokaryotic expression systems as well as use of fusion proteins were used in these studies mentioned. Moreover, antimicrobials expressed in a simple *E. coli* host will probably have low yields as *E. coli* growth may be inhibited by the expressed proteins (Doherty et al. 1993, Chen et al. 2008). The purification process was nevertheless efficient for isolating the desired protein, as SDS-PAGE analysis showed that there was a single band corresponding to the expected size of 8.35 ± 3 kDa (Figure 4.6) indicating that the protein had been purified successfully in an active form.

The polyhistidine-tag used during expression is one of the most popular affinity tags used as an efficient tool for purifying proteins (Hochuli et al. 1987, Hochuli 1988, Lichty et al. 2005). It allows efficient purification of tagged proteins in high yields without any prior knowledge of their biochemistry, and has a minimal effect on the tertiary structure and biological activity of the protein (Arnau et al. 2006). It can be subsequently eluted from the resin by competition with free imidazole. The removal of the affinity tag is controversial and principally depends on the downstream use of the purified proteins. More than 60% of proteins produced for structural studies include a polyhistidine tag (Derewenda 2004). Affinity tags could either improve target protein

properties or lower yields and enzyme activity (Cadel et al. 2004, Arnau et al. 2006, Kou et al. 2007). It was chosen not to remove the affinity tag after purification of *palG*1 due to economical and practical reasons, for example the total yields and time saving resulting from the low numbers of chromatographic steps. To assess whether the protein functions are affected by the His-tag, activity of recombinant *palG*1 protein could be compared to untagged proteins and the positive control protein included in the expression kit to see if the activity levels are different. Structural models could also be used to ascertain whether the tag affects the protein structure or not. This method relies on comparative methods using existing models of previously crystallized proteins as templates and the low similarity of the protein sequences identified here could potentially cause a problem.

In conclusion, out of 11 genes identified in the two cow rumen metagenomic libraries, only one gene, *palG*1 could be successfully expressed and purified in sufficient yields for downstream applications. It may be necessary to attempt expression of the other identified AMPs in other *E. coli* expression systems (pET-22b(+) or using fusion technology described above. However, fusion technology methods require that the AMP be separated from its fusion partner, and recombinant fusion proteins, including multimeric ones, are usually cleaved with enzymes such as furin or chemicals such as cyanogen bromide (CNBr) (Lee et al. 2002, Kim et al. 2008), which results in inefficient cleavage and thus poor recovery of AMPs from fusion partners, as well as unwanted amino acid residue(s), which can decrease antimicrobial activity and cause problematic side effects (Li et al. 2005). Irrespective, it is still possible to express novel AMPs from natural environments using the ptrchisTOPO in *E. coli* TOP10 cells as demonstrated for *palG*1.

CHAPTER FIVE

Antimicrobial activity and mode of action studies of novel antimicrobial genes identified from the rumen

5.1 Introduction and aim of chapter

In Chapters three anf four, a number of novel antimicrobial genes were identified from the IBERS fosmid based cow rumen metagenome library (Prive 2011) and from an already existing cow rumen metagenome (Hess et al. 2011) using both functional and sequence based metagenomic techniques, as well as, other bioinformatic and computational platforms. Five long (>22 AAs) antimicrobial genes (Gene 6, 17A, 17B *palG*1 and *palG*2) were identified from the in-house metagenome library, including 181 short antimicrobial peptides. Six genes H-G1, H-G2, H-G3, H-G4, H-G5, and H-G6 were identified from the (Hess et al. 2011) dataset. Of these, only *palG*1 was successfully amplified and expressed in a suitable *E. coli* host. Also, a peptide library of the short AMPs containing 135 peptides (≤22 AAs) was chemically synthesized. H-G2 and H-G4 (22 AAs) were also chemically synthesized.

This chapter describes the antimicrobial susceptibility and activity screens of the antimicrobial proteins identified above in this study, as well as various mode of action studies to understand the killing mechanisms of the AMPs against pathogenic bacteria.

5.2 Materials and methods

5.2.1 Antimicrobial screen and analysis of peptide library from fosmid-based cow rumen metagenomic library

The activity of the 135 peptides in the peptide library (section 3.3.1.3) was screened by a high throughput method using both luminescent and non-luminescent bacterial strains described in section 5.2.1.1 and 5.2.1.2 below

5.2.1.1 Antimicrobial activity screen of peptide library against luminescent *P. aeruginosa* strain H1001

The antimicrobial activity of peptides in the synthesized library against a luminescent *P. aeruginosa* strain H1001 (Lewenza et al. 2005) was performed as described by (Hilpert and Hancock 2007). A single colony of *P. aeruginosa* strain H1001 from a freshly made Mueller Hinton (MH) agar plate was inoculated into a sterile tube containing 3 ml MH followed by overnight incubation at 37^{o}C in a shaker at 225 rpm. Fifty microlitres of the overnight culture (1:100 dilution) was added to 4,950 µl MH broth in a tube, and incubated in a shaker at 37^{o}C at 225 rpm until the OD_{600}nm reached 0.35 (approximately 2 hours). Thereafter, 1,920 µl of the microbial culture was added to 48,000 µl solution A (100 mM Tris–HCl buffer, pH 7.3, 20 mM glucose) to make solution B. This amount of solution B is sufficient for ten complete 96-well plates. Solution B was then vortexed gently for 2 minutes and the assay was set up in a clear, sterile, U shaped polypropylene 96-well plate (Greiner Bio One Ltd, Stonehouse, UK) (Figure 5.1) as follows. Solution B (110 µl) was transferred into each of the 12 wells of row A. The luminescence signal of the bacteria in these wells was tested using a Tecan infinite F200 pro Quad4 Monochromator micro-plate reader (Tecan Group AG, Switzerland) at excitation/emission spectra of 470/695 nm to avoid wasting peptides in case the bacteria do not display any luminescence. If a luminescence signal was detected (at least more than 900 light units), 70 µl of solution B was added into each of the 12 wells of rows B-F, rows G_{2-6} and rows H_{1-6}, while 110 µl was added wells G_1. Wells G_{7-12} and H_{7-12} were empty. Thirty microlitres of the prepared peptide solution (from punched cellulose membrane from spot synthesis) were then transferred into the corresponding 12 wells of row A of the 96-well plate and well G_1 (140 ml in total in these wells). In other words, peptides 1–12 were added to the corresponding wells in row A, positive control peptide is added to well G_1. The control peptide

(KRRWRIWLV) used was a peptide synthesised in the same run as the other peptides being tested. Its MIC was predetermined, as it had been previously synthesized on resin No peptide is added to the wells in row H. This serves as the growth/negative control to obtain 100% luminescence value for the untreated microbes. The contents of row A was thoroughly mixed using a 12-channel multipipette and 70 µl was transferred into each corresponding well of row B. This stepwise dilution series was continued until row F. A serial dilution of the positive control peptide was also carried out. After mixing the solution in the last wells of the dilution series, 70 µl was discarded so that the total volume in all wells was 70 µl. The luminescence of the plate is again measured and then incubated at 37^{o}C for 4 hours, after which a final luminescence reading is taken. Results obtained were thereafter analysed using the MATLAB extension Gait-CAD (section 5.2.1.3).

In addition, 70 µl MH medium was added into each well and plates were sealed and incubated at 37^{o}C for 16–20 h or when satisfactory growth is obtained (wells show visible turbidity). Wells in which no microbial growth has occurred are determined visually. Alternatively, OD_{600}nm of the entire plate can be taken. By comparing the luminescence data with these growth data, it is possible to identify those peptides whose activity is strongly influenced by cation concentration (e.g. magnesium) as well as those peptides with killing abilities over a longer period of time. It should be noted that there was no change observed in activity for any of the peptides whether after 4 hours or after an additional 16-20 hour incubation.

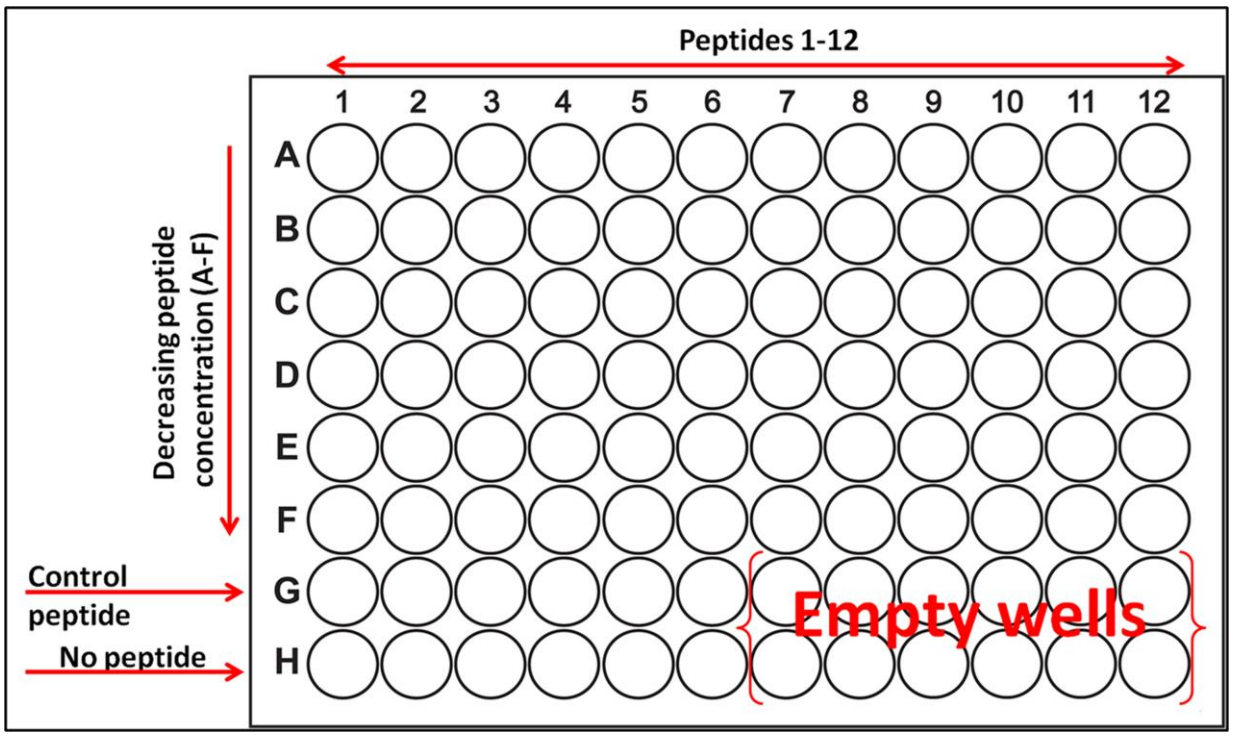


Figure 5.1 Arrangement of 96 well plate in peptide library screens.

5.2.1.2 Antimicrobial activity screen of peptide library against non-luminescent bacterial strains

The peptide library was also screened for activity against non-luminescent bacteria strains including EMRSA-15, *Sal. typhimurium* and *E. coli* K12. Overnight cultures were prepared and diluted to yield a bacterial concentration of 5 x 10^5 CFU/ml (Friedrich et al. 2000). Into a black, opaque, polypropylene 96 well plate (Greiner Bio One Ltd, Stonehouse, UK) 110 µl of culture was added in all wells of the first row A and the first well of row G. Into the last 5 wells of row G, 70 µl of sterile MH was added to act as a negative control. Into all other empty wells (except wells $G_{7\text{-}12}$ and $H_{7\text{-}12}$, which were empty), 70 µl of bacteria culture in MH broth (5 x 10^5 CFU/ml) was added. Into wells 1-12 of row A and well G_1, 30 µl of peptides in solution (from spot synthesised peptides) was added. A two-fold serial dilution was then carried out as described in section 5.2.2.1 above. The control peptide used was the same as in the section 5.2.2.1 and has a previously determined MIC against MRSA of 16 µg/ml. The

first 6 wells of row H contained bacteria but no drug, this served as a measure of maximum growth.

The plates were then covered and incubated for 4 hours at 37°C, after which 14 µl of 500 µM resazurin in sterile, demineralised H_2O was added to each well (100 µm final concentration). The plates were then returned to the incubator for an additional hour, after which fluorescence readings of each well was measured using a Tecan infinite F200 pro Quad4 Monochromator micro-plate reader (Tecan Group AG, Switzerland) at excitation/emission spectra of 560/590 nm. The results were then analysed using the MATLAB extension Gait-CAD (section 5.2.2.3).

5.2.1.3 Activity analysis of luminescence and fluorescence dilution series for peptides in antimicrobial peptide library against pathogenic bacteria

MATLAB analysis was carried out in collaboration with Prof Ralf Mikut at Karlsruhe Institute for Technology (KIT), Germany. Data obtained from dilution series for activity of 135 peptides (including positive and negative controls) against *P. aeruginosa*, EMRSA-15, *E. coli* K12 and *Sal. typhimurium* were imported into Gait-CAD (Mikut et al. 2008) separately for each Excel table. Concentration values with a 75% reduction of luminescence or fluorescence (IC75) in relation to the control were computed to identify artifacts of positive and negative controls (Mikut 2010). Fuzzy features were also computed according to Mikut and Hilpert (2009). Then all the projects (activity of 135 peptides against all four pathogens) were fused to get the overview for all peptides. The analysis was then based on IC75 values instead of relative values to positive control (RelIC75) as pre-analysis showed some problems with positive controls (positive control peptide showed the same killing activity on all plates for the same organism but not across organisms) (Hilpert and Hancock 2007, Mikut 2010). The minimum, mean and median values of IC75 against all four microorganisms were computed. This was

followed by the computation of three activity classes: active (IC75≤0.25), semi-active (0.25<IC75≤0.50) and inactive (IC75>0.5) for the minimum, mean and median values against all microorganisms. IC75, RelIC75 and derived values were saved into an Excel table (see Table 5.1 in CD/Figshare), and finally, amino acid distribution and hydrophobicity vs. charge were visualized (Mikut and Hilpert 2009) (see Figure 5.3 in section 5.3).

5.2.2 Determination of minimum inhibitory concentration (MIC) of selected antimicrobial proteins

Minimal inhibitory concentrations (MICs) were determined using two-fold serial dilutions of antimicrobial agents in MH broth as described by the National Committee of Clinical Laboratory Standards (NCCLS) (NCCLS, 1997). The MICs for antimicrobial peptides and comparator antimicrobials were measured using a modified broth microdilution method in MH broth. Briefly, the peptides were dissolved and stored in sterile polypropylene tubes. The assay was performed in sterile U bottom 96 well polypropylene microplates with lids (Greiner Bio One Ltd, Stonehouse, UK). Serial dilutions of antimicrobials were performed in sterile distilled water at 10-fold the desired final concentrations. Ten microlitres of this 10 fold dilutions were added to each well of a 96 well polypropylene microplates containing 90 µl of MH broth per well. Bacteria from overnight culture (adjusted to 1×10^8 CFU/ml) were added to the wells of the plate (final inoculum concentration of 5×10^5 CFU/ml) (Cherkasov et al. 2008, Wiegand et al. 2008). The MIC was defined as the lowest concentration of drug that inhibited visible growth of the organism after an 18-24 hour incubation at appropriate temperatures.

5.2.3 Erythrocyte leakage assay

Erythrocyte leakage assay using defribinated sheep blood (Oxoid Ltd Hampshire, UK), was used to determine whether antimicrobial peptides would cause erythrocyte lysis, and therefore possible cytotoxicity. Briefly, six sterile microcentrifuge tubes were each filled with 1 ml fresh defibrinated sheep red blood cells (RBC) and centrifuged for 5 minutes at 1,000 x g in a sci spin mini centrifuge in a microbiology safety cabinet. The supernatant was discarded and the pellet washed 3 times with phosphate buffered saline (35 mM PBS) (pH 7.3). Centrifugation at 1,000 x g was carried out between each wash. The red blood cells were then diluted in PBS to 4% v/v in one vial (8.6 ml total volume was used per 12 peptides to be tested/per 96 well plate). Thereafter, 90 µl of the 4% solution was added to all wells in the plate. Ten micro litres of peptides (at 10X desired final concentration) were added to RBC solution in all wells of columns 1-9 of all rows on plate, mixed through pipetting and serially diluted down the rows. Wells in column 10 contained RBC with triton X-100 (Sigma-Aldrich UK) at different concentrations (1, 0.8, 0.6, 0.4, 0.2, 0.1, 0.05% and 0.025%), while wells in column 11 contained RBC with 0.1% triton-X which served as a positive control (0.1% causes 100% cell lysis). The serial dilution of the triton-X in column 10 provided an added control to identify any differences between plates. Column 12 contained RBC with no peptide as this was the negative control. Plates were then covered and left for 1 hour in a 37°C incubator, after which they were centrifuged for 5 minutes at 1,000 x g in a refrigerated centrifuge. After centrifugation, 70 µl of supernatant from each well of the plate was then transferred to a transparent, flat bottom 96 well plate and the absorbance (OD_{450}nm) of the supernatant was measured using the Hidex Sense Plate Reader Software 0.5 pre-release (LabLogic, Sheffield UK) or the Synergy™ H4 Hybrid Multi-Mode reader (BioTek Swindon UK) to detect any haemoglobin that had leaked from the erythrocyte cytoplasm. The results obtained were used to determine the percentage haemolysis

given that the 0.1% triton-X represented 100% lysis, the negative control was taken as background. Experiments were performed in quadruplicates at sub-MIC (0.5 X MIC), MIC and supra-MIC (2 X MIC and 4 X MIC) concentrations.

5.2.4 Time kill assays

5.2.4.1 Time kill assay for synthesized antimicrobial peptides

The growth kinetics or rate of kill of pathogens by peptides assay was adapted from (Oliva et al. 2003). Again, the assay was performed in 96 well plates. In summary, overnight cultures were prepared as stated previously and an optical density measurement (OD_{600}nm) taken to provide an estimate of cell numbers in the culture. To ensure the bacteria were in logarithmic growth phase a 1:25 dilution was carried out on the culture using MH broth and the culture was then left for 2 hours shaking at 37^0C. A second optical density measurement was taken and compared to a previously determined standard for the organism that related optical density to colony forming units (CFU) per ml. This provided a rough quantification of the cells in culture and the amount of MH broth required to dilute the culture to 1 x 10^6 CFU/ml was added.

The bacterial suspension (1 x 10^6) (180 µl) was added to all wells of the first row A of the 96 well plate. The antimicrobial compounds to be tested were then added to columns 1-11 of the same row at a final concentration of 3 times the MICs. No drug was added to column 12, which served as negative control and depicted a "healthy" bacterial growth curve. The plate was then placed in a 37^0C shaking incubator. The culture used for the assay was also serially diluted (1:10) in 100mM Tris buffer pH 7.5 and each dilution plated onto MH agar plates. At time points: 10, 20, 30, 40, 50, 60, 90, 120, 150, 180, 210, 240, 300, 360 and 1440 minutes, 5 µl of culture containing the antimicrobial (in 96 well plate) was removed from each well, serially diluted (1:10) in 100mM Tris buffer pH 7.5, and each dilution was plated onto MH agar. The agar plates

were then incubated for 15 hours at 37^0C before CFU/ml was calculated. Experiments were performed in quadruplicates.

5.2.4.2 Time kill assay for *palG*1

Due to low expression and yield of *palG*1 *in vivo* (highest concentration being 400 µg/ml), it was impossible to measure the rate of kill against the susceptible pathogen (*Ent. faecalis*), at 3 X MIC concentration as described for synthetic AMPs above. The time course of cell killing by *palG*1 was therefore calculated by optical density measurements. An increase both in cell mass and cell number can readily be estimated by measuring the turbidity of a cell suspension using a spectrophometer, thereby offering a rapid and sensitive alternative to cell counting (Hobson et al. 1996, Dalgaard and Koutsoumanis 2001, Prescott et al. 2004, Madrid and Felice 2005). This method has been shown to produce comparable results to plate counting, flow cytometric and green fluorescence viability analysis methods (Lehtinen et al. 2006). In a 96 well plate, *palG*1 at MIC concentration (200 µg/ml) was added to cells in mid-logarithmic phase (1×10^6 OD_{600}nm of ≤ 0.2) in LB broth and serially diluted as previously described. Plates were incubated at 37^0C in a microplate incubator shaker. Cells with no drug were taken as growth controls. The rate of kill was calculated as a percentage (OD_{600}nm) of surviving cells (percentage of viable cells was equal to 100% for the control (cells without antibiotics)) over a 24 hour period (Lehtinen et al. 2006, Hazan et al. 2012).

5.2.5 Inner membrane depolarisation assay (diSC3(5)

To measure the ability of peptides to disrupt the electrochemical potential across the bacterial cytoplasmic membrane, the release of the membrane-associated probe, 3,3′-dipropylthiadicarbocyanine iodide [$diSC_3(5)$], from *E. coli* K12 and EMRSA-15 cells was monitored by fluorescence spectroscopy as described previously (Wu et al. 1999, Lee et al. 2004). The dye quenches its own fluorescence once inside the cytoplasmic

membrane. Upon addition of a peptide, dye is released into the aqueous medium (if the membrane is depolarized i.e. inside becomes less negative), resulting in increased fluorescence (Sims et al. 1974).

The assay was carried out in 96 well plates. Briefly, mid-logarithmic phase bacteria cells (OD_{600}nm of 0.2) were washed with 5 mM HEPES, pH 7.2, 5 mM glucose buffer, and resuspended to an OD_{600}nm of 0.05 in the same buffer. The cell suspension was incubated with 100 mM Potassium chloride (KCl) and 0.4 mM 3,3′-dipropylthiadicarbocyanine iodide [$diSC_3(5)$] until a stable reduction of fluorescence was achieved (approximately 1 hour). The KCl was added to equilibrate the cytoplasmic and external K^+. After 1 hour, 90 µl of appropriate bacterial cell suspension was added to all wells in columns 1-12. Peptides (10 µl) prepared at 10X the desired final concentration ((3 X MIC) except for *palG*1 (MIC)) were then added to corresponding wells in columns 1-10 and mixed thoroughly. Wells in column 11 contained Triton X-100 (to give 0.1% final concentration) as positive control, while wells in column 12 were untreated. The plate was incubated at 37°C with shaking and the fluorescence was continuously monitored (excitation λ 622 nm, emission λ 670 nm) upon addition of peptide at 2 to 5 minute intervals for 20 to 30 minutes.

5.2.6 Transmission electron microscopy

Overnight cells were cultured in Mueller-Hinton (MH) broth to mid-log phase and harvested by centrifugation at 1,000 x g for 10 minutes. Cell pellets were washed twice with 10 mM PBS and resuspended to an OD_{600}nm of 0.2. The cell suspension (1 ml) was incubated at 37°C for 60 minutes with different peptides at a concentration of 3 X MIC in microcentrifuge tubes. After incubation, the cells were centrifuged and washed 3 times at 5,000 x g for 5 minutes with PBS. These were then sent in sealed microcentrifuge tubes to the Advanced Microscopy and Bio-Imaging Laboratory at

IBERS. Embedding and examination of treated bacterial samples by transmission electron microscopy were carried out with the assistance of Mr Alan Cookson and Dr Susan Girdwood. The protocol used is described below.

All timed steps involving fixatives, washes, ethanol mixtures and resin infiltration were conducted on a suitable rotator at room temperature in a fume hood unless otherwise stated. Centrifugation was always for 5 minutes at 10,000 rpm. The entire contents of the microcentrifuge tubes were mixed with 1 ml of a primary fixative (2.5% glutaraldehyde in 0.1 M sodium cacodylate at pH 7.2 both from Agar Scientific Ltd). In the meantime, a 2% ultra-low gelling temperature agarose solution (Sigma Aldrich Dorset, UK Cat. No. A-5030) was made up in ultra-pure H_2O. This was dissolved at 50^0C, cooled and filtered with a 0.22 µm syringe filter (Whatman) and kept at 25^0C until use. After 30 minutes fixation, the samples were centrifuged and the supernatant discarded. The pellets were re-suspended in another 1 ml of fresh fixative as above for a further 30 minutes, followed by centrifugation. Thereafter, the pellets were re-suspended in 1 ml 0.1 M sodium cacodylate wash buffer pH at 7.2. The samples were centrifuged and the supernatant discarded. They were re-suspended in 1 ml of a secondary fixative (1% osmium tetroxide (Agar Scientific Ltd. Essex, UK) made up in 0.1 M sodium cacodylate buffer pH at 7.2). After 30 minutes fixation, the samples were centrifuged and the supernatant was carefully discarded and replaced with a rinse in 1 ml of wash buffer as above for 5 minutes and were again centrifuged and supernatant discarded. This step was repeated twice. Following the wash steps, the sample pellets were re-suspended in 100 µl agarose solution at 25^0C and placed in a refrigerator to gel at 4^0C.

After gelling overnight, the agarose containing the treated bacteria samples was cut from the microcentrifuge tubes and transferred into 1 ml wash buffer in 5 ml glass vials

with push-on lids at 4^0C. After 30 minutes, the gelled agarose pellets were placed in fresh wash buffer. The samples were then progressed through an alcohol series of 30%, 50%, 70%, 95% and three changes of 100% for at least an hour. Afterwards, they were transferred to a 1:2 mixture of ethanol to LR White-hard grade resin (London Resin Company Ltd. London, UK), then a 2:1 mixture of ethanol to resin and finally 100% resin overnight at 4^0C. The next morning, the resin was removed and replaced with fresh resin and later that day, the samples were placed in size 4 gelatine moulds (Agar Scientific Ltd. Essex, UK), filled up with fresh resin and polymerised overnight in an oven at 60ºC.

Thick sections (2 µm), which contained the bacteria were cut and dried down on drops of 10% ethanol on glass microscope slides. They were stained with Ammonium molybdate (AMB) stain and photographed using a Leica DM6000B microscope. Ultrathin sections (60–80 nm) were then cut on a Reichert-Jung Ultracut E Ultramicrotome with a Diatome Ultra 45 diamond knife and collected on Gilder GS2X0.5 3.05 mm diameter nickel slot grids (Gilder Grids, Grantham, UK), float-coated with Butvar B98 polymer (Agar Scientific Ltd. Essex, UK) films. All sections were double-stained with uranyl acetate (Agar Scientific Ltd. Essex, UK) and Reynold's lead citrate (TAAB Laboratories Equipment Ltd, Aldermaston, UK) and observed using a JEOL JEM1010 transmission electron microscope (JEOL Ltd, Tokyo, Japan) at 80 kV. The resulting images were photographed using Kodak 4489 electron microscope film (Kodak Ltd, Hemel Hempstead, UK), developed in Kodak D-19 developer for 4 minutes at 20°C, fixed, washed and dried according to the manufacturer's instructions. The resulting negatives were scanned with an Epson Perfection V800 film scanner and converted to positive images.

5.2.7 Molecular modelling of peptide 3D structures

Molecular modeling was done with the help of Dr Narcis Fernandez-Fuentes, IBERS Aberystwyth University.

The modeling of the 3D conformation of peptides was carried out using algorithms from the PEP-FOLD program (web server http://bioserv.rpbs.univ-paris-diderot.fr/PEP-FOLD/ (Maupetit et al. 2009, Maupetit et al. 2010), a *de novo* approach aimed at predicting peptide structures from amino acid sequences. PEP-FOLD is based on Monte Carlo simulation that explores the assembly of fragments derived for each peptide. Firstly, a limited number (200) of local conformations at each position of the structure is predicted using a Hidden Markov Model-derived structural alphabet (HMM-SA) (Camproux et al. 2004). Subsequently, the assembly is performed using a greedy procedure (Tuffery and Derreumaux 2005, Tuffery et al. 2005), driven by a coarse-grained energy score using the Optimal Potential for Efficient Peptide-Structure Prediction (OPEP) force-field (Maupetit et al. 2007). The best 3D models for each peptide were selected according to PEP-FOLD score, considering the lowest energy model (sOPEP, score Optimized Potential for Efficient structure Prediction). The three top scoring models were selected for each sequence. Given the intrinsic nature of peptides that are highly heterologous in conformation, it is better to consider an ensemble rather than the lowest energy conformation (Saladin et al. 2014). However, the structural modeling of *palG*1, a longer polypeptide (71 AAs), i.e. a mini protein, was done using the PHYRE2 web portal http://www.sbg.bio.ic.ac.uk/phyre2 (Kelley and Sternberg 2009, Kelley et al. 2015). The method and approaches used above are briefly described below. Results from all modeling were visualized using PyMOL v1.7.6 program (Schrödinger 2010).

5.2.8.1 PEP-FOLD: a *de novo* peptide structure prediction tool

PEP-FOLD (Maupetit et al. 2009, Maupetit et al. 2010, Thevenet et al. 2012) is a *de novo* structural modelling of peptides between 9 and 25 residues in free form. By using a HMM-SA library of 27 structural 4-mers, PEP-FOLD assigns the more likely structural fragment for a given amino acid sequence (Camproux et al. 2004). Subsequently, the fragments are assembled into peptides using a greedy procedure driven by a modified version of the OPEP coarse-grained force field (Tuffery and Derreumaux 2005, Tuffery et al. 2005, Maupetit et al. 2007). Given an amino-acid sequence, PEP-FOLD performs series of 50 simulations and returns the most representative conformations identified in terms of energy and population. Under benchmark conditions, PEP-FOLD was able to predict successfully the conformation of 52 peptides between 9–23 residues long with a root-mean-square deviation (RMSD) between 2.8 and 2.3 Å (considering Cα only), outperforming previous approaches. In the case of 13 miniproteins, i.e. between 27–49 residues long, PEP-FOLD reached an accuracy of 3.6 and 4.6 Å Cα RMSD considering the lowest-energy conformations. PEP-FOLD simulations are fast (a few minutes only) opening therefore, the door to *in silico* large-scale rational design of new bioactive peptides and miniproteins. PEP-FOLD has also been used in other research projects to predict biologically relevant peptide structures (Duvignaud et al. 2010, Kawaguchi et al. 2010, Steckbeck et al. 2011).

5.2.8.2 The Hidden Markov model derived structural alphabet (HMM SA) for proteins

An important aspect of PEP-FOLD is the use of a fragment library composed of structural fragments. The fragments derive from a structural alphabet: HMM-SA composed of 27 structural prototypes 4-mers called structural letters, allowing the simplification of the 3D protein structures into 1D sequences of structural letters

(Camproux et al. 2004, Regad et al. 2011). The method for predicting SA letters using HMM is described in Maupetit et al. (2010). It has been shown that HMM-SA is an effective and relevant tool for the study of protein structures, to study protein contacts, or protein deformations, to search for 3D similarity across proteins, to predict the conformation of peptides in aqueous solutions, and to extract structural motifs from protein loops (Guyon et al. 2004, Regad et al. 2006, Martin et al. 2008, Martin et al. 2008, Regad et al. 2008, Maupetit et al. 2009, Maupetit et al. 2010, Regad et al. 2010, Baussand and Camproux 2011).

5.2.8.3 The Optimal potential for efficient peptide-structure prediction (OPEP) force field

The search and scoring of peptide conformation is done against a force-field. PEP-FOLD utilizes the OPEP force-field (Maupetit et al. 2007), which is a coarse-grained protein model based on a six-bead model per residue (hydrogens not considered). OPEP uses a detailed representation of all backbone atoms (N, H, Cα, C and O) and reduces each side-chain to one single bead with appropriate geometrical parameters and van der Waals radius. The implicit solvent OPEP energy function is expressed as a sum of local potentials (taking into account the changes in bond lengths, bond angles, improper torsions of the side-chains and backbone torsions), non-bonded potentials (taking into account the hydrophobic and hydrophilic properties of each amino acid) and hydrogen-bonding potentials (taking into account two- and four- body interactions) (Nasica-Labouze et al. 2011). The potential function was parameterized to differentiate native and non-native (or decoys) structures and in relation to the lowest free energy (Kar et al. 2013). The OPEP force field has been used in several protein studies (Chebaro and Derreumaux 2009, Chebaro et al. 2012) and extensively tested on peptides using multiple approaches such as the activation-relaxation technique, Monte Carlo, Molecular Dynamics (MD) and Replica Exchange Molecular Dynamics (REMD)

simulations as well as greedy-based algorithms (Maupetit et al. 2009, Maupetit et al. 2010).

5.2.8 Analysis of variance (ANOVA) and *post-hoc* tests

Two-way Analysis of variance (ANOVA) with factors 'antimicrobial treatments' and 'time' using Microsoft Excel was performed in order to determine whether there were significant changes in cell viability and membrane depolarisation in relation to time, before and after peptide treatments (Harmon 2011). Results obtained from the ANOVA were then verified by *post-hoc* multiple comparisons using Tukey's HSD (Honestly Significant Difference) Studentized test. The Tukey's *post-hoc* test is useful for calculating simultaneous confidence intervals for all pairwise differences between means in a balanced design, especially with large group sizes (≥4 groups); accurately maintaining alpha (α) levels at their intended values (Jaccard et al. 1984, Tukey et al. 1985, Wright 1992, Bender and Lange 2001, Feise 2002) Alpha (α) levels were set at $P < 0.05$. This method is fully described by Harmon (2011).

5.3 Results

5.3.1 Analysis of antimicrobial activity screen of peptide library

Activity analysis of highthroughput screen of the 135 peptides in the peptide library against luminescent and non-luminescent bacteria pathogens (see Figure 5.2 for examples of 96 well plates from this screen) using the MATLAB GAIT_CAD software revealed that some peptides had some activity against all microorganisms with the best activity against *E. coli* K12. Table 5.2 is a summary of the number of peptides per organism for different activity classes.

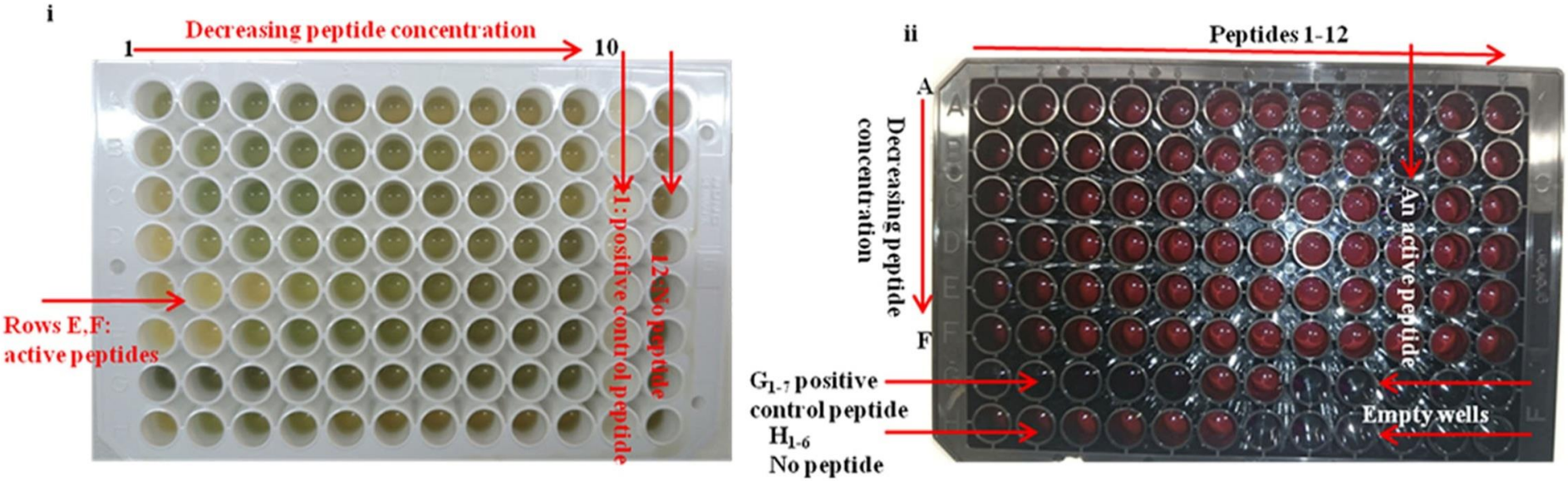


Figure 5.2 96 well plate showing antimicrobial activity screen. **i.** luminescent, **ii.** non-luminescent pathogens

Table 5.2 Summary of activity class of peptides against pathogens

Pathogen	Active IC75≤0.25	Semi-active 0.25<IC75≤0.50	Inactive IC75>0.5
Pseudomonas aeruginosa H1001	3	11	121
EMRSA-15	0	6	129
E. coli K12	11	6	118
Salmonella typhimurium	2	6	127
MEAN	2	5	128
MEDIAN	2	5	128
MINIMUM	12	13	110

In total, 25 peptides had activity against at least one pathogen tested as shown on Table 5.3 (see Table 5.1 in CD/Figshare for complete activity analysis data of all 135 peptides).

Mean and median values of 2 peptides indicate a good average activity against all pathogens, the best ones being LPRRNRWSKIWKKVVTVFS and HLRRINKLLTRIGLYRHAFG (peptides 2 and 3).

Some peptides had selective activities, e.g. SIKILKIYFIQGKRHWSF (peptide 12) against *P. aeruginosa* H1001. Amino acids like W, R, F, T tended to improve activity while amino acid C tended to decrease activity (see Figure 5.3). As in previous studies (Mikut and Hilpert 2009, Ramon-Garcia et al. 2013), a similar pattern of hydrophobicity vs. positive charge balance, i.e., a "yin-yang balance" was observed. A low proportion of unloaded and non-hydrophobic AAs also seemed to be an advantage as shown in Figure 5.4.

Table 5.3 IC75 values of most active peptides (Green = active, yellow = semi active, and uncolored means inactive).

S/N	Peptide number in library	Peptide sequence	*E. coli* K12 dilution series IC75	EMRSA-15 dilution series IC75	*Sal. typhimurium* dilution series IC75	*P. aeruginosa* H1001 dilution series IC75	Mean IC75 for all pathogens	Median IC75 for all pathogens	Min IC75 for all pathogens
1	69	THRLRRWCRARGLAR	0.05	1.03	1.04	0.71	0.71	0.87	0.05
2	124	LPRRNRWSKIWKKVVTVFS	0.06	0.38	0.24	0.14	0.21	0.19	0.06
3	58	HLRRINKLLTRIGLYRHAFG	0.09	0.29	0.09	0.14	0.15	0.11	0.09
4	110	VLHTGYRKFLHRSKRFFHLR	0.09	0.32	0.27	0.43	0.28	0.30	0.09
5	100	TMSLRFWRWKVR	0.11	0.55	0.44	0.62	0.43	0.49	0.11
6	111	LTKKTKKQKRNLVGTT	0.12	1.15	0.36	0.39	0.50	0.37	0.12
7	91	NRFTARFRRTPWRLCLQFRQ	0.16	0.65	0.41	0.54	0.44	0.47	0.16
8	108	AWRWKAFRNCWRVRSSSL	0.17	0.78	3.81	0.40	1.29	0.59	0.17
9	86	LIRCSRTCLQYKTSRFMRW	0.19	1.15	0.79	0.58	0.68	0.68	0.19
10	48	RSITRPVLVRRRWRVRPVF	0.20	0.68	0.48	0.61	0.49	0.55	0.20
11	128	RICRTRLTRRAGNSL	0.22	0.93	0.63	1.69	0.86	0.78	0.22
12	135	SIKILKIYFIQGKRHWSF	303.61	11.53	17.09	0.25	83.12	14.31	0.25
13	22	QVRWWGRYWRRKWATCR	0.31	0.33	0.46	0.31	0.35	0.32	0.31
14	80	MRILSIIRWTRMKKSSA	0.62	0.44	0.82	0.31	0.55	0.53	0.31
15	15	GTAWRWHYRARS	0.34	0.89	0.67	0.78	0.67	0.72	0.34
16	72	VGVKRRLKCLLSLRS	0.34	7.65	1.02	0.45	2.37	0.73	0.34
17	47	RRLRTTTKLPPV	0.35	1.99	0.73	1.24	1.08	0.98	0.35
18	87	TTAPCKCWIGLRRCFK	0.36	3.77	1.99	1.55	1.92	1.77	0.36
19	70	RLLLVMIGLRSKIKWHSGI	0.72	0.96	10000000000	0.39	2500000000	0.84	0.39
20	133	THILLLRLRKKVMS	0.63	0.40	0.70	0.67	0.60	0.65	0.40
21	109	SRATWARVRRLGLYG	0.41	0.84	0.69	0.85	0.70	0.76	0.41
22	96	KFVRLKIYCRDKNKGRGISF	0.64	5.34	7.79	0.43	3.55	2.99	0.43
23	68	AVWMTRSCVIWKR	0.69	2.48	6.98	0.47	2.66	1.59	0.47
24	105	LLMRKLIKGYGYLFGKGKRKKR	0.62	0.71	1.30	0.47	0.78	0.67	0.47
25	76	MAKLLRLDKKRNKFLCFV	1.02	11.03	4.91	0.49	4.36	2.97	0.49

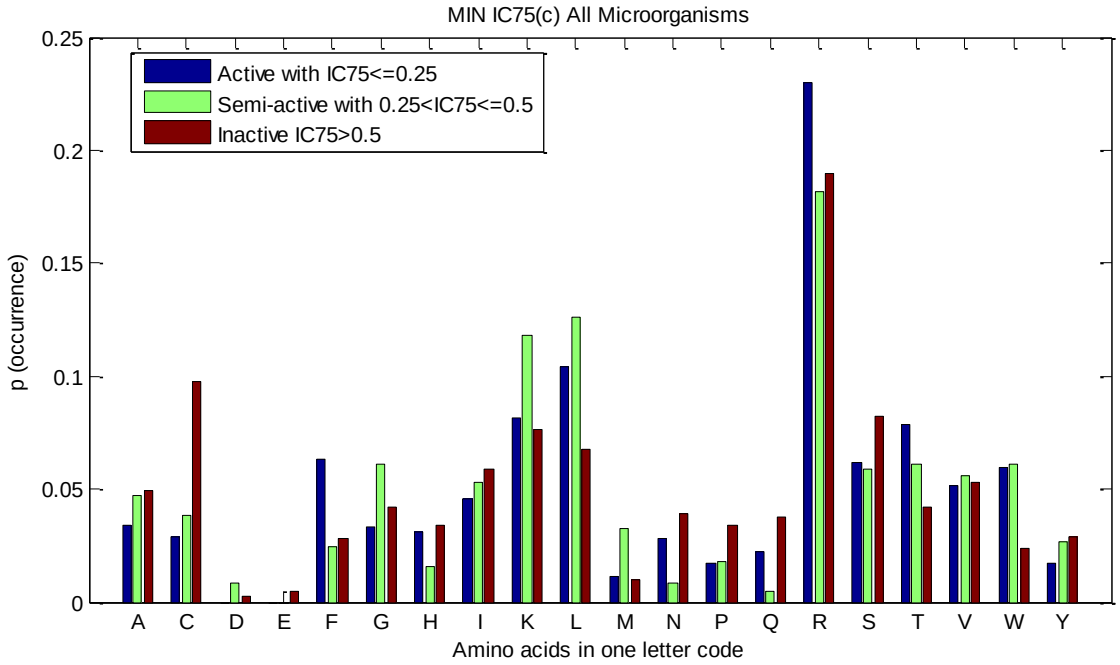


Figure 5.3 Visualization of amino acid (AA) distributions for activity classes (based on minimum IC75 for all microorganisms), color-coded bars for AA distributions.

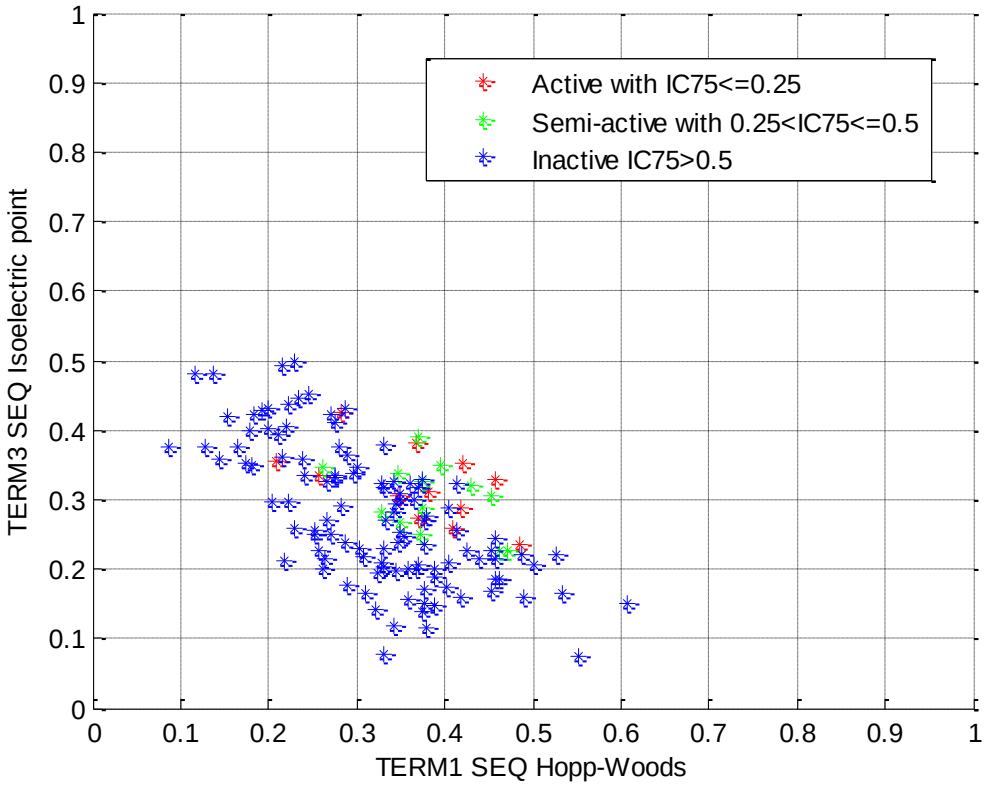


Figure 5.4 Hydrophicity (measured by TERM1 SEQ Hoop-Woods) vs. Positive Charge (measured by TERM3 SEQ Isoelectric point) for activity classes (Mikut 2010), red points mean high activity against at least one microorganism (IC75≤0.25), blue means inactivity against all microorganisms (IC75>0.5), green points represent semi-active peptides (0.25<IC75≤0.5).

Based on this analysis, 15 peptides, peptides 1-10, 12, 13, 19, 20 and 22 (Table 5.3) were selected for further analysis as they had higher activity ranking, broad-spectrum and or specific activity against pathogens. They were reassigned numbers - peptides 1-15. These 15 peptides were then synthesized by (GenScript USA Inc. New Jersey, USA) at >95% purity on resin using solid phase Fmoc chemistry as described in sections 2.7.2 and 2.7.4. All peptides were linear and C-terminal amidated (see 'Genscript 1' in CD/Figshare for Mass Spectometry and HPLC information for all resin synthesized peptides).

5.3.2 Minimum inhibitory concentration (MIC) of all identified antimicrobial peptides

The MIC was defined as the lowest concentration of drug that inhibited visible cell growth of the organism after an 18-24 hour incubation at appropriate temperatures. Cell growth was defined as a visible clot in an individual well, regardless of its size (Kim et al. 2015). Therefore, the MICs determined using this method would be more stringent (accounting for at least a 2-fold increase in MIC) than those determined using the Sensititre™ system (Saini et al. 2011) which defines growth as the generation of a clot following antibiotic treatment that is similar in size to the clot generated in the absence of antibiotic. The determined MICs values outlined in Table 5.4 below were reproducible in duplicates within three different experiments.

Table 5.4 Minimum inhibitory concentration values of identified antimicrobial peptides (n = 6) nt (not tested), > (precedes the highest concentration tested).

		Minimum inhibitory Concentrations MICs (µg/ml)						
Peptide ID	**Peptide Sequence**	***P. aeruginosa***	***Sal. typhimurium***	***E. coli***	***EMRA-15***	***S. aureus***	***Ent. faecalis***	***List. monocytogenes***
1	THRLRRWCRARGLAR-NH_2	250	250	125	250	250	250	31.25
2	LPRRNRWSKIWKKVVTVFS-NH_2	31.25	31.25	31.25	31.25	31.25	31.25	31.25
3	HLRRINKLLTRIGLYRHAFG-NH_2	125	31.25	62.5	31.25	31.25	31.25	31.25
4	VLHTGYRKFLHRSKRFFHLR-NH_2	500	31.25	62.5	62.5	125	62.6	62.5
5	TMSLRFWRWKVR-NH_2	62.5	62.5	62.5	31.25	62.5	62.5	62.5
6	LTKKTKKQKRNLVGTT-NH_2	>1000	>1000	>1000	>1000	>1000	>1000	>1000
7	NRFTARFRRTPWRLCLQFRQ-NH_2	31.25	62.5	62.5	31.25	31.25	62.5	31.25
8	AWRWKAFRNCWRVRSSSL-NH_2	31.25	31.25	62.5	31.25	62.5	62.5	62.5
9	LIRCSRTCLQYKTSRFMRW-NH_2	500	62.5	250	125	250	500	125
10	RSITRPVLVRRRWRVRPVF-NH_2	500	62.5	250	31.25	250	500	500
11	SIKILKIYFIQGKRHWSF-NH2	500	500	250	125	250	250	250
12	QVRWWGRYWRRKWATCR-NH_2	500	250	250	250	250	250	125
13	RLLLVMIGLRSKIKWHSGI-NH_2	500	250	250	250	250	250	250
14	THILLLRLRKKVMS-NH_2	500	500	250	500	500	500	500
15	KFVRLKIYCRDKNKGRGISF-NH_2	125	125	62.5	62.5	125	125	125
Control peptide	KRRWRIWLV-NH_2	15.6	7.81	7.81	15.6	7.81	15.6	15.6
Hess Gene 2 (H-G2)	MKKLLLILFCLALALAGCKKAP-NH_2	>1024	>1024	>1024	>1024	>1024	>1024	>1024
Hess Gene 4 (H-G4)	VLGLALIVGGALLIKKKQAKS-NH_2	≥1000	512	512	32	128	128	512
Bactenecin 2A (Bac2A)	RLARIVVIRVAR-NH_2	256	128	128	64	64	64	64
Polymyxin B sulphate		1.95	1.95	1.95	250	250	31.25	62.5
Ciprofloxacin		0.49	0.12	0.06	>250	>250	62.5	62.5
Vancomycin hydrochloride		62.5	250	125	0.98	0.98	62.5	62.5
*palG*1		nt	>200	>200	nt	>200	200	nt

MICs of some peptides (2, 3, 4, 5, 7, 8, 9, 10 and 15) showed similar activity spectra and confirmed broad spectrum activity observed in the peptide library screen with MIC range of 32 to 64 µg/ml across all pathogens. Other peptides (1, 6, 11, 12, 13, and 14) had higher MICs (>64 µg/ml). AMPs also showed antimicrobial activity against *Ent. faecalis* and *List. monocytogenes*, which were not included in the initial screen. Peptide H-G2 and H-G4 were predicted to be AMPs using computational methods and had not been previously tested for activity against pathogens. H-G2 seemed not to have any activity against all pathogens tested. This may be because H-G2 was synthesized as a linear peptide instead of a cyclic peptide as it has equal number of cysteine residues and may form disulphide bonds. H-G4 on the other hand, showed some activity against Gram positive but not Gram negative organisms, and seems to have specific activity against EMRSA-15 (MIC 32 µg/ml). *palG*1 was only active against *Ent. faecalis* with an MIC of 200 µg/ml. It also showed minimal inhibition of *E. coli* growth (observed in growth curves) with no detectable MIC at the highest concentration tested. This may account for the low level of expression of *palG*1. The highest concentration of *palG*1 tested was 200 µg/ml due to low levels of protein expression and/or yield of purified protein.

Based on MIC results, eight peptides (2, 3, 4, 5, 7, 8, 10, and 15) with broad spectrum activity were chosen for further activity and mode of action studies. *E. coli* K12 and EMRSA-15 were chosen as representative organisms for Gram negative and Gram positive organisms respectively.

5.3.3 Erythrocyte leakage assay

The haemolytic activity of antimicrobial peptides against erythrocytes is often used as a measure of their cytotoxicity to eukaryotic cells and to estimate their therapeutic index. The haemolysis or erythrocyte leakage induced by antimicrobial peptides was calculated

as a percentage using the formula: haemolysis (%)=[($\mathbf{A}_{450}$ in treated supernatant−$\mathbf{A}_{450}$ in PBS)/($\mathbf{A}_{450}$ in 0.1% Triton X-100−$\mathbf{A}_{450}$ in PBS)]×100. All experiments were performed in quadruplicates and the results represent means ± standard deviations of four independent experiments. Figure 5.5 is a chart showing % erythrocyte leaked of all peptides at varying concentrations.

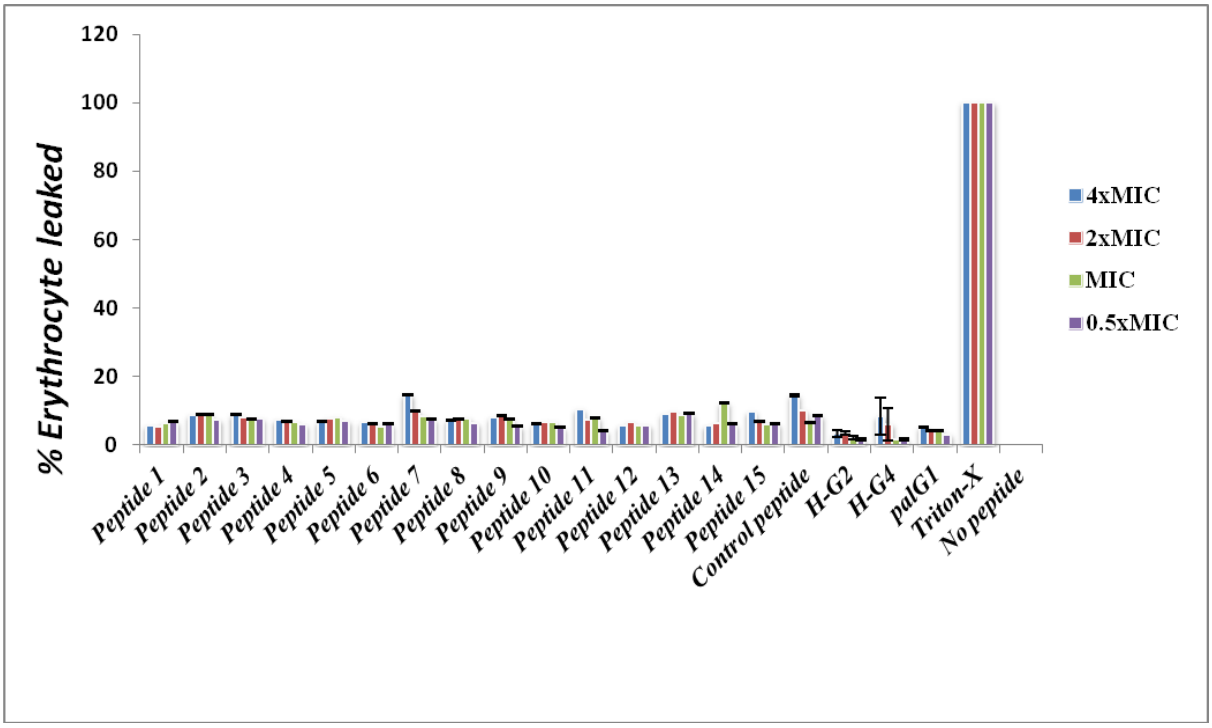


Figure 5.5 Haemolytic activities of peptides at sub-MIC, MIC and supra-MIC concentrations (values from four independent replicates; error bars represent one standard deviation).

All peptides had little haemolytic effect on sheep blood, with only around 10% haemolysis even at 4 X MIC concentrations (2 X MIC for *palG1*).

5.3.3 Time kill assay

5.3.3.1 Time kill assay for synthesized antimicrobial peptides

Time kill assays were performed to determine the rate of antimicrobial peptides induced cell death against EMRSA-15 and *E. coli* K12 (see Figures 5.6 and 5.7). The killing activities of the selected peptides (2, 3, 4, 5, 7, 8, 10, and 15) with broad spectrum activity were measured in MH broth. Colonies on MH agar plates were counted and

viability was calculated as the number of colony forming units per millilitre of culture. Results are from four independent replicates and error bars represent standard deviations from the mean.

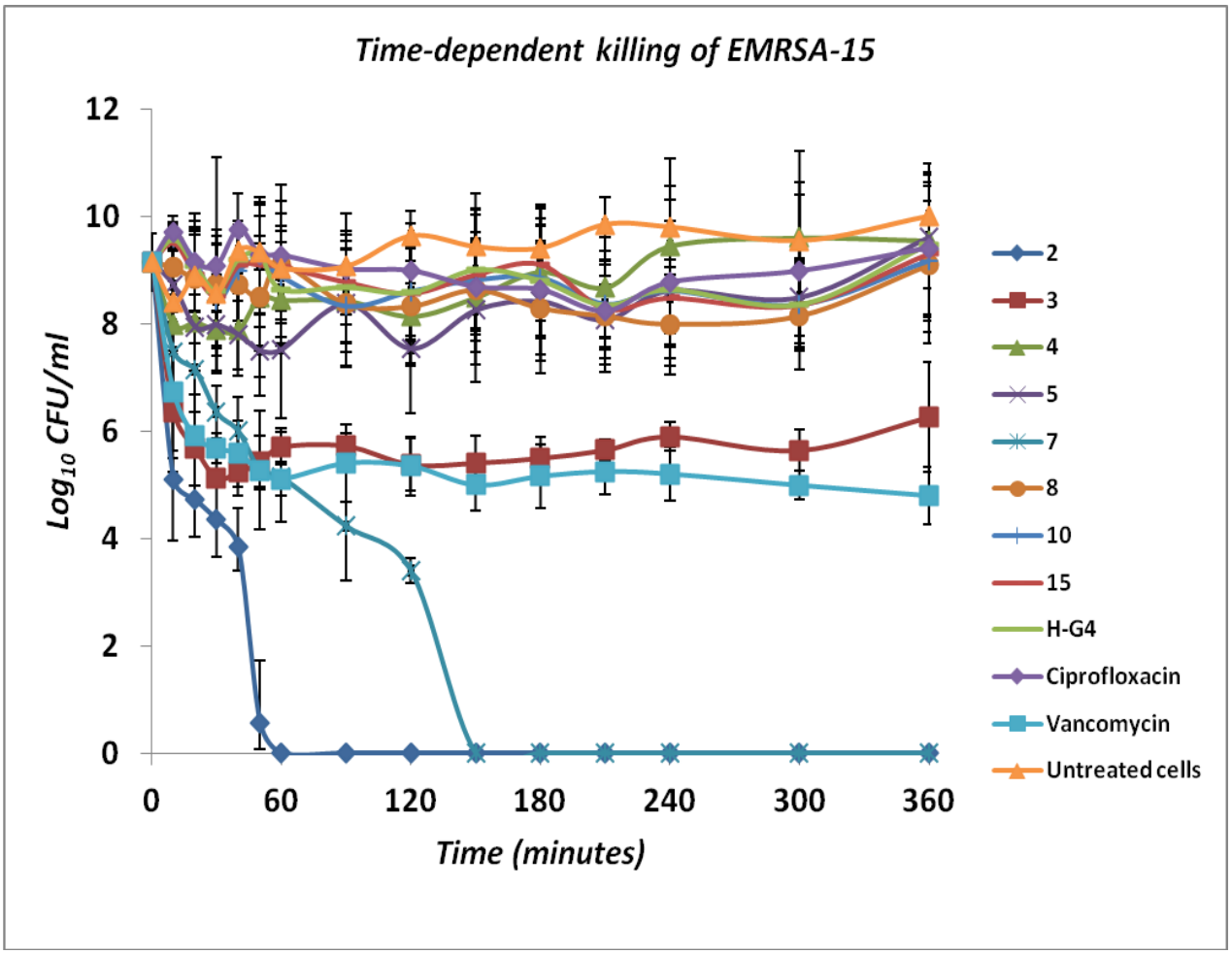


Figure 5.6 Time dependent kill of EMRSA-15 by peptides (values from four independent replicates; error bars represent one standard deviation).

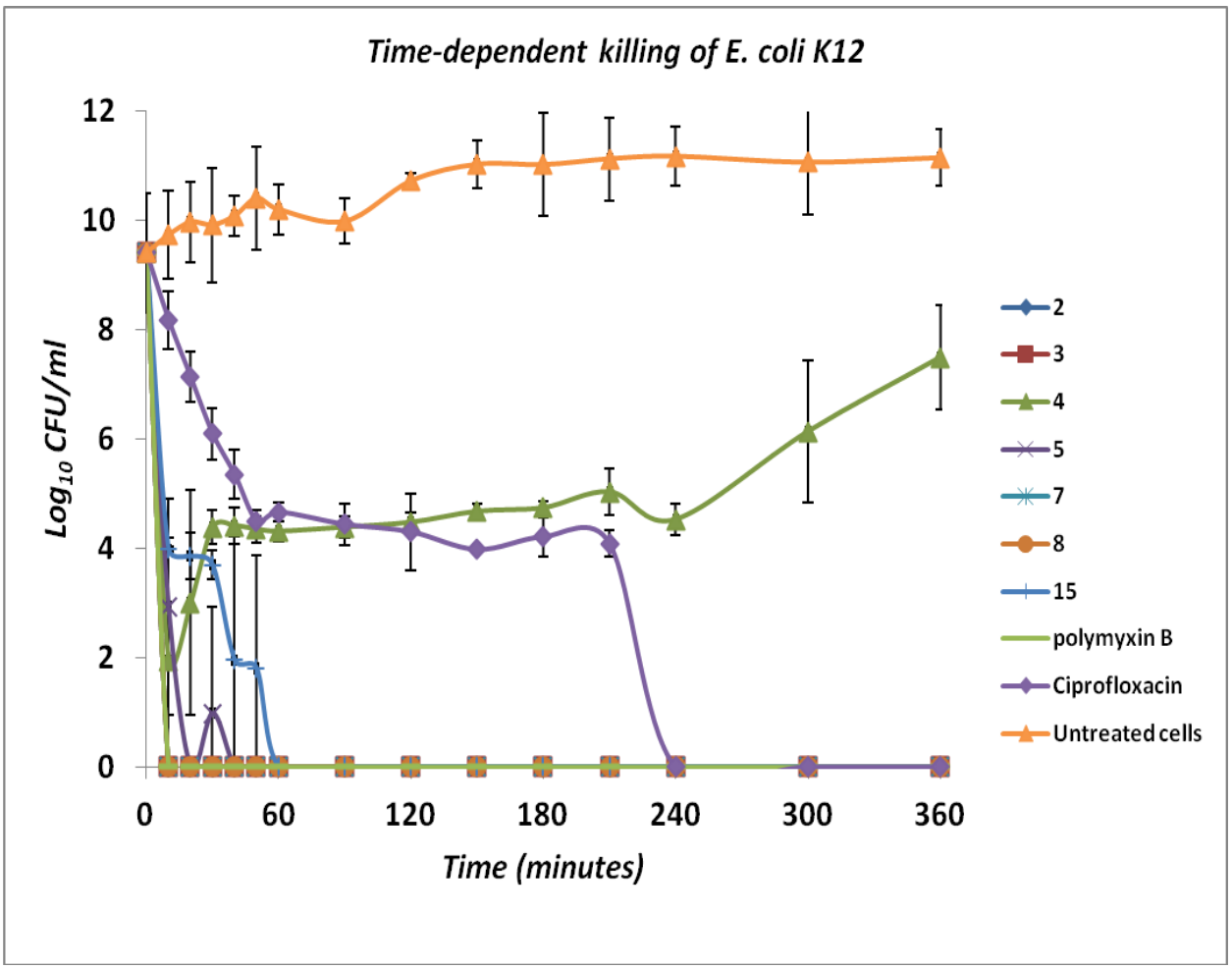


Figure 5.7 Time dependent kill of *E. coli* K12 by peptides (values from four independent replicates; error bars represent one standard deviation).

There was a significant difference between peptide treated and untreated *E. coli* K12 cells ($P < 0.001$). All peptides triggered a rapid decline in *E. coli* K12 viability with complete cell death within 10 minutes (for peptides 2, 3, 7, 8) and 1 hour for peptides 5 and 15. Interestingly, although peptide 4 caused > 5 log reduction in viability in the first 30 minutes, cells seemed to recover after 4 hours of incubation. Polymyxin B and ciprofloxacin showed expected killing activities against *E. coli* K12. In contrast, although there was a significant difference between peptide treated and untreated EMRSA-15 cells ($P < 0.05$), the peptides generally showed lower activity against EMRSA-15. No bactericidal activity was observed in EMRSA-15 cells treated with peptides 4, 5, 8, 10, 15 and H-G4 (≤ 2 log reduction in viability). Peptide 2, and 7 had

the most activity against EMRSA-15 causing rapid kill and complete cell death within 1 and 3 hours respectively. Peptide 3 had comparable killing activity to Vancomycin with ≥ 4 log decrease in cell viability. Ciprofloxacin had no activity against EMRSA-15 as expected. Tukey's *post-hoc* tests also show that loss of viability was significantly higher in peptides 2 and 7, Vancomycin and peptides 3 in that order (q = 63.477, 48.452, 29.173 and 31.207). Table 5.5 below provides a summary of q and $q_{critical}$ values obtained from *post-hoc* tests. The folder 'statistical analysis' in CD/Figshare contains all ANOVA and Tukey's *post-hoc* tests for all multiple comparisons (combinations of pairs).

5.3.3.1 Time kill assay for *palG*1

Figure 5.8 shows the killing activity of *palG*1 against *Ent. faecalis*, calculated as a percentage (OD_{600}nm) of surviving cells.

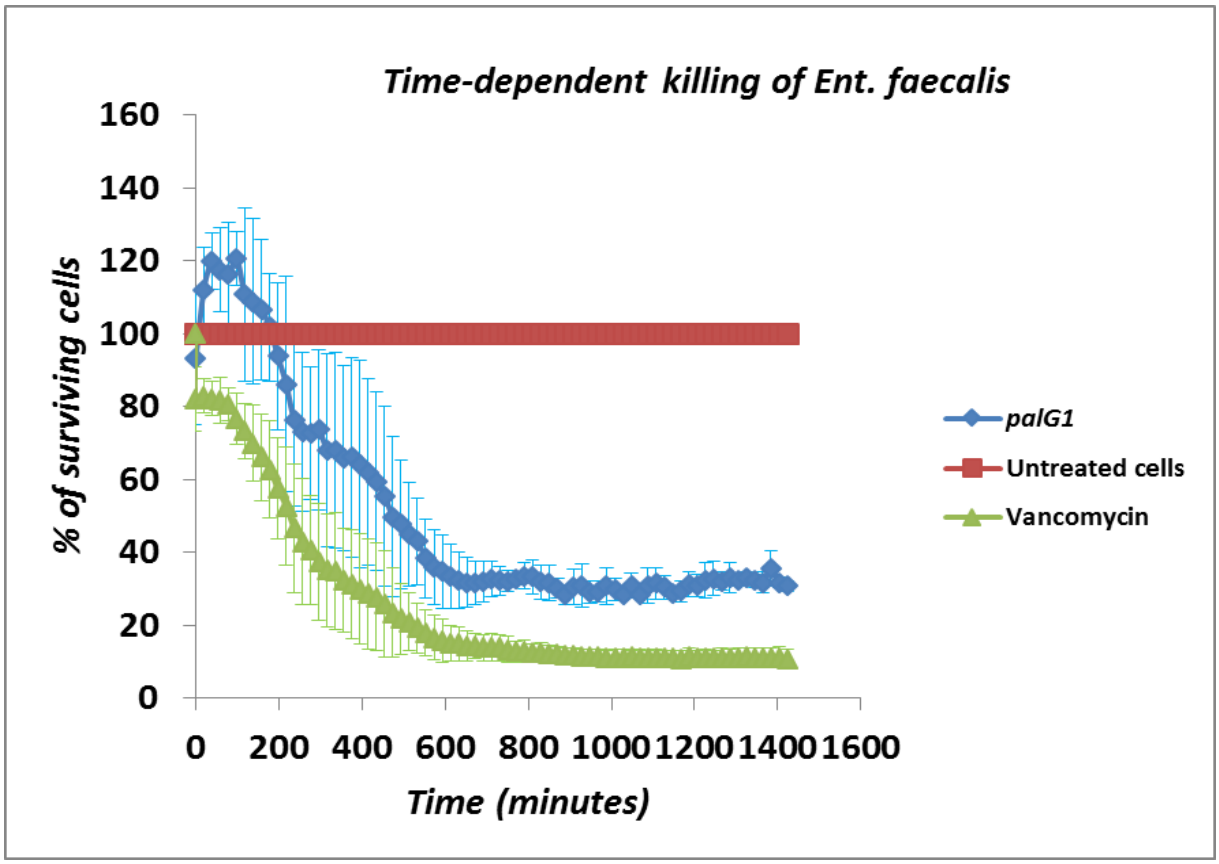


Figure 5.8 Time dependent kill of *Ent. faecalis* by *palG*1 (values from four independent replicates; error bars represent one standard deviation).

As can be seen from Figure 5.8 above, *palG*1 had a steady kill with only ≤30% of surviving cells after 24 hours. Tukey's *post-hoc* test showed that the treated (vancomycin and *palG*1 treated) cells had significantly higher loss of viability than the untreated *Ent. faecalis* cells (Table 5.5).

5.3.4 Inner membrane depolarisation

AMP induced membrane depolarisation in *E. coli* K12 and EMRSA-15 is shown in Figure 5.9 and 5.10 respectively. *palG*1 induced membrane depolarisation in *Ent. faecalis* is shown in Figure 5.11. Error bars represent the standard deviations from the mean from three different experiments.

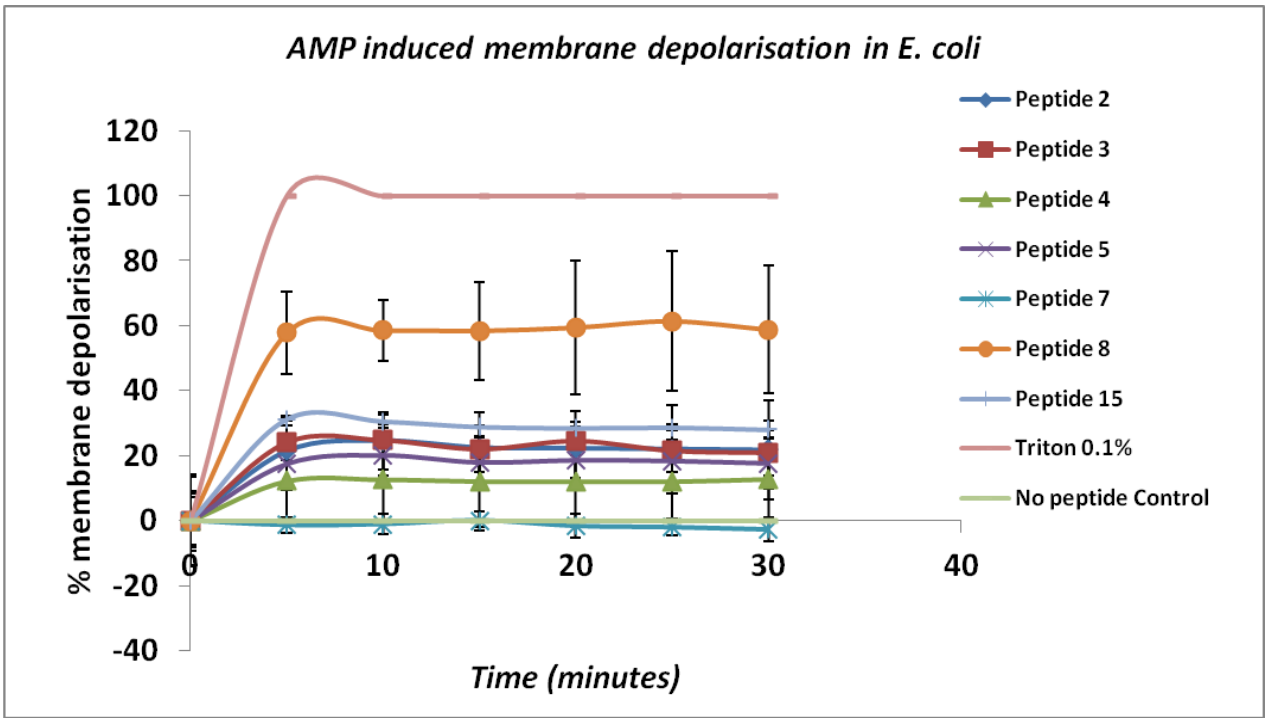


Figure 5.9 AMP induced membrane depolarisation in *E. coli* K12 (values from three independent replicates; error bars represent one standard deviation).

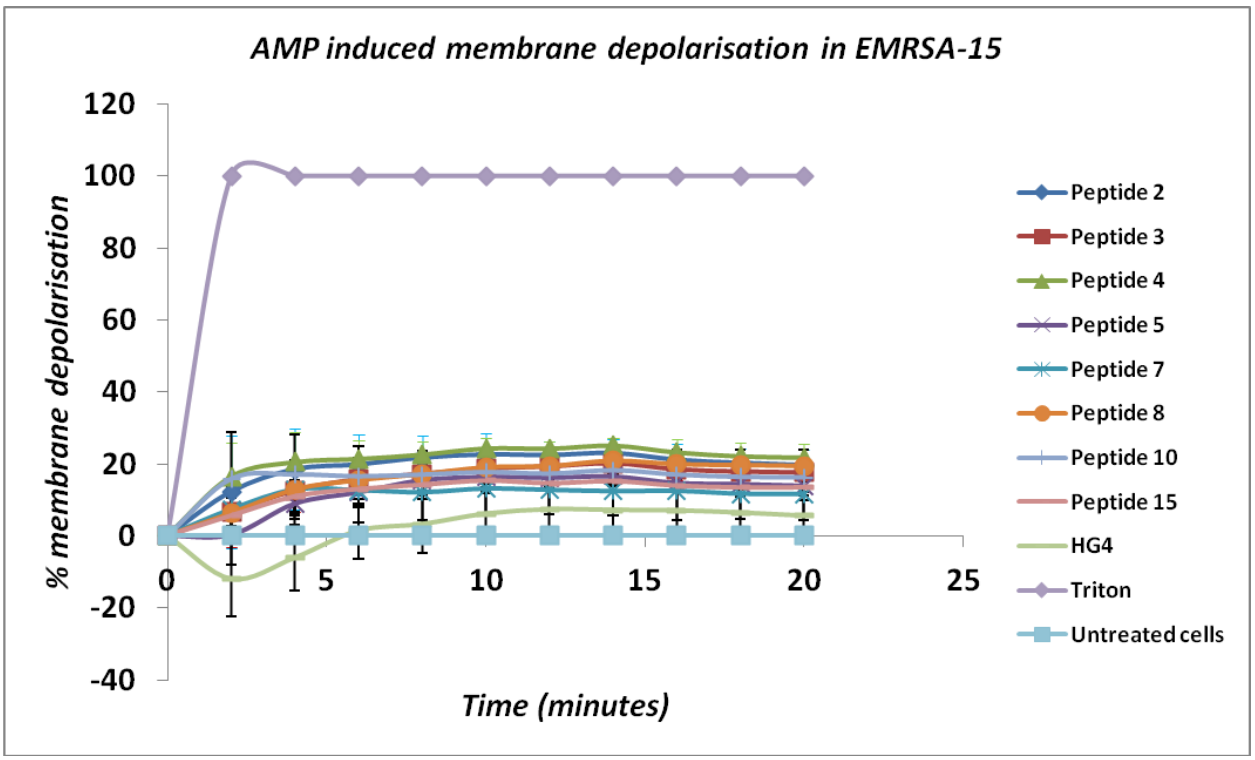


Figure 5.10 AMP induced membrane depolarisation in EMRSA-15 (values from three independent replicates; error bars represent one standard deviation).

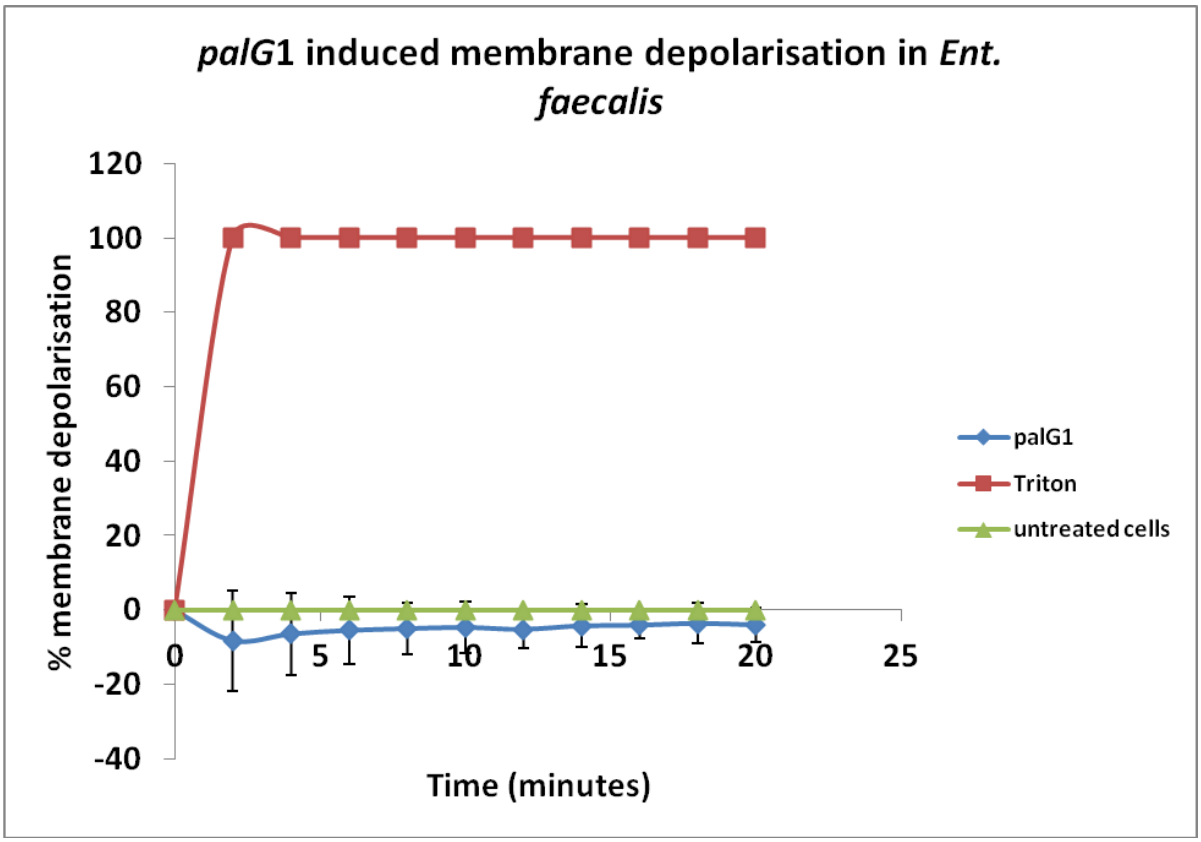


Figure 5.11 *palG*1 induced membrane depolarisation in *Ent. faecalis* (values from three independent replicates; error bars represent one standard deviation).

Generally all peptides had some membrane depolarisation effect against both organisms tested with around 10-30% depolarisation (Figures 5.9 and 5.10). Tukey's *post-hoc* test revealed that peptide treatments induced significantly higher membrane depolarisation of *E. coli* K12 (Table 5.5 - all q values < $q_{Critical}$). Peptide 8 had the most membrane depolarisation effect (>50% depolarisation) against *E. coli* K12 (Figure 5.9). Tukey's *post-hoc* test also showed that peptides had no significant depolarisation effect on EMRSA-15 membrane (all q values < $q_{Critical}$ - Table 5.5). Although there were significant differences between treatments (*palG*1, triton, and untreated cell), *palG*1 did not induce membrane depolarisation in *Ent. faecalis* in the short time of this assay (Figure 5.11).

Table 5.5 Summary of Tukey's HSD *Post-hoc* test and pair wise multiple comparisons: α = 0.05, Asterisks (*****) indicate significantly different groups. There is a significant difference between group means if **q > q $_{Critical}$**, (TRUE) at α level of 0.05 and vice versa (FALSE).

Level comparisons	Full description of comparisons	Mean of pairs compared	MS_{within}	n	Absolute difference in means of pair	q	$q_{Critical}$	Significance
			Time Kill assays					
			EMRSA-15 (ANOVA, *P* = 2.3486E-272)					
1,2	untreated vs pep2*****	8.880	1.891	0.776	64.000	6.989	63.477	4.635	TRUE
1,3	untreated vs pep3*****	8.880	5.668	0.776	64.000	3.212	29.173	4.635	TRUE
1,4	untreated vs pep4*****	8.880	8.236	0.776	64.000	0.644	5.851	4.635	TRUE
1,5	untreated vs pep5*****	8.880	7.912	0.776	64.000	0.968	8.793	4.635	TRUE
1,6	untreated vs pep7*****	8.880	3.545	0.776	64.000	5.335	48.452	4.635	TRUE
1,7	untreated vs pep8*****	8.880	8.229	0.776	64.000	0.651	5.909	4.635	TRUE
1,8	untreated vs pep10	8.880	8.440	0.776	64.000	0.439	3.992	4.635	FALSE
1,9	untreated vs pep15	8.880	8.482	0.776	64.000	0.398	3.612	4.635	FALSE
1,10	untreated vs H-G4	8.880	8.497	0.776	64.000	0.383	3.481	4.635	FALSE
1,11	untreated vs Ciprofloxacin	8.880	8.667	0.776	64.000	0.213	1.936	4.635	FALSE
1,12	untreated vs Vancomycin*****	8.880	5.444	0.776	64.000	3.436	31.207	4.635	TRUE
3,12	pep3 vs Vancomycin	5.668	5.444	0.776	64.000	0.224	2.034	4.635	FALSE
			***E. coli* (ANOVA, *P* = 0, i.e. P < 0.001)**					
1,2	untreated vs pep2*****	9.965	0.744	0.419	64.000	9.221	113.918	4.496	TRUE
1,3	untreated vs pep3*****	9.965	0.744	0.419	64.000	9.221	113.918	4.496	TRUE
1,4	untreated vs pep4*****	9.965	4.733	0.419	64.000	5.232	64.635	4.496	TRUE
1,5	untreated vs pep5*****	9.965	0.988	0.419	64.000	8.977	110.902	4.496	TRUE
1,6	untreated vs pep7*****	9.965	0.744	0.419	64.000	9.221	113.918	4.496	TRUE
1,7	untreated vs pep8*****	9.965	0.744	0.419	64.000	9.221	113.918	4.496	TRUE
1,8	untreated vs pep15*****	9.965	1.699	0.419	64.000	8.266	102.123	4.496	TRUE
1,9	untreated vs Ciprofloxacin*****	9.965	4.302	0.419	64.000	5.663	69.965	4.496	TRUE
1,10	untreated vs Polymyxin B*****	9.965	0.744	0.419	64.000	9.221	113.918	4.496	TRUE
			***Ent. faecalis* (ANOVA, *P* =3.5495E-279)**					
1,2	untreated vs *palG*1*****	100.000	31.666	80.418	222.000	68.334	113.538	3.314	TRUE
1,3	untreated vs Vancomycin*****	98.676	11.234	80.418	222.000	87.441	145.284	3.314	TRUE
2,3	*palG*1 vs Vancomycin*****	31.666	11.234	80.418	222.000	20.431	33.947	3.314	TRUE

Inner membrane depolarisation (diSC3(5)) assays

EMRSA-15 (ANOVA, *P* = 7.57519E-29)									
1,2	untreated vs pep2	0.167	0.334	0.086	36.000	0.168	3.436	4.552	FALSE
1,3	untreated vs pep3	0.167	0.304	0.086	36.000	0.137	2.807	4.552	FALSE
1,4	untreated vs pep4	0.167	0.352	0.086	36.000	0.185	3.801	4.552	FALSE
1,5	untreated vs pep5	0.167	0.275	0.086	36.000	0.108	2.215	4.552	FALSE
1,6	untreated vs pep7	0.167	0.266	0.086	36.000	0.099	2.030	4.552	FALSE
1,7	untreated vs pep8	0.167	0.309	0.086	36.000	0.142	2.914	4.552	FALSE
1,8	untreated vs pep10	0.167	0.307	0.086	36.000	0.140	2.876	4.552	FALSE
1,9	untreated vs pep15	0.167	0.275	0.086	36.000	0.108	2.213	4.552	FALSE
1,10	untreated vs H-G4	0.167	0.188	0.086	36.000	0.022	0.447	4.552	FALSE
1,11	untreated vs Triton*****	0.167	1.000	0.086	36.000	0.833	17.081	4.552	TRUE
***E. coli* (ANOVA, *P* = 7.67871E-81)**									
1,2	untreated vs pep2*****	0.250	17.021	54.212	24.000	16.771	11.159	4.427	TRUE
1,3	untreated vs pep3*****	0.250	17.815	54.212	24.000	17.565	11.687	4.427	TRUE
1,4	untreated vs pep4*****	0.250	9.306	54.212	24.000	9.056	6.026	4.427	TRUE
1,5	untreated vs pep5*****	0.250	13.927	54.212	24.000	13.677	9.100	4.427	TRUE
1,6	untreated vs pep7	0.250	-0.788	54.212	24.000	1.038	0.691	4.427	FALSE
1,7	untreated vs pep8*****	0.250	44.024	54.212	24.000	43.774	29.125	4.427	TRUE
1,8	untreated vs pep15*****	0.250	22.465	54.212	24.000	22.215	14.781	4.427	TRUE
1,9	untreated vs Triton*****	0.250	75.250	54.212	24.000	75.000	49.902	4.427	TRUE
***Ent. faecalis* (ANOVA, *P* =1.38502E-78)**									
1,2	untreated vs *palG*1*****	0.167	-4.092	16.885	72.000	4.258	8.793	3.377	TRUE
1,3	untreated vs Triton*****	0.167	83.500	16.885	72.000	83.333	172.083	3.377	TRUE
2,3	*palG*1 vs Triton*****	-4.092	83.500	16.885	72.000	87.592	180.876	3.377	TRUE

5.3.5 Transmission electron microscopy

TEM was carried out to determine whether the loss of viability in EMRSA-15, *E. coli* K12 and *Ent. faecalis* following exposure to peptides was a result of, or accompanied by cell morphological and cell wall ultrastructure changes. Figures 5.12, 5.13 (i and ii) and 5.14 show TEM images obtained for the different peptide treatments. Magnification scale of each micrograph is indicated below each image.

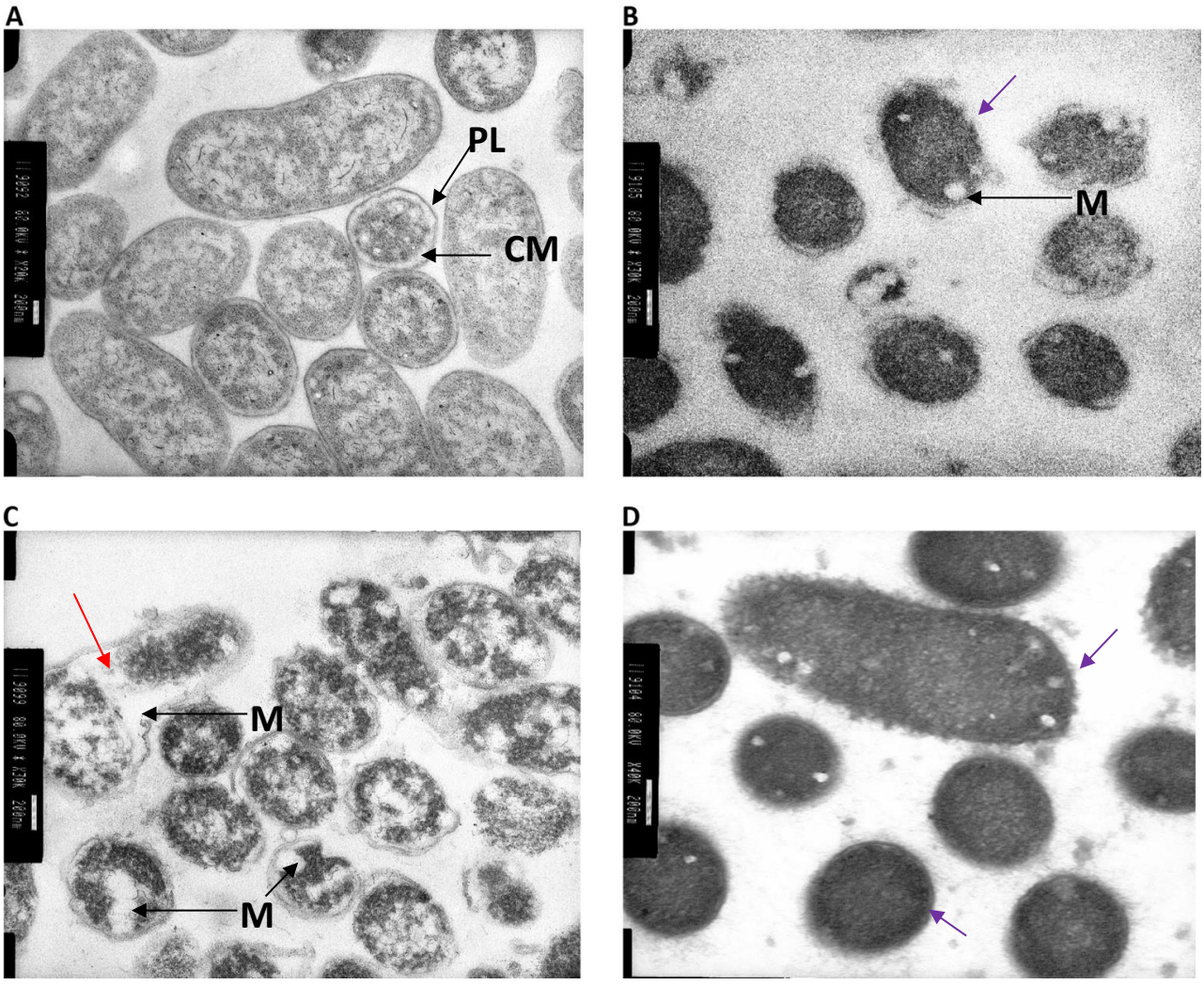


Figure 5.12 TEM images of EMRSA-15 - following 60 minutes (A) Untreated (B) exposure to peptide 2 (C), exposure to peptide 3 and (D) exposure to peptide 7 at 3 X MIC concentrations. PL = Peptidoglycan layer, CM = cytoplasmic membrane, M = Mesosomes. Scale bars are 200nm. Magnification is 20,000X (A), 30,000X (B and C) and 40,000X (D).

TEM images show that compared to untreated EMRSA-15 cells, which had intact cell morphology (Figure 5.12 A), peptide treated cells induced notable mesosome-like structures and defects in the cell wall (Figures 5.12 b, C D). Outer cell detachment and spilling off of the cytoplasmic content can be seen in cells treated with peptide 3 (Figure 5.12 C - red arrow). The cell wall also appears to be severely damaged and shows undulations. Cell shrinkage and numerous protrusions (a 'hairy' appearance) were observed in EMRSA-15 cells treated with peptides 2 and 7 (figure 5.12 B and D - purple arrows).

(i)

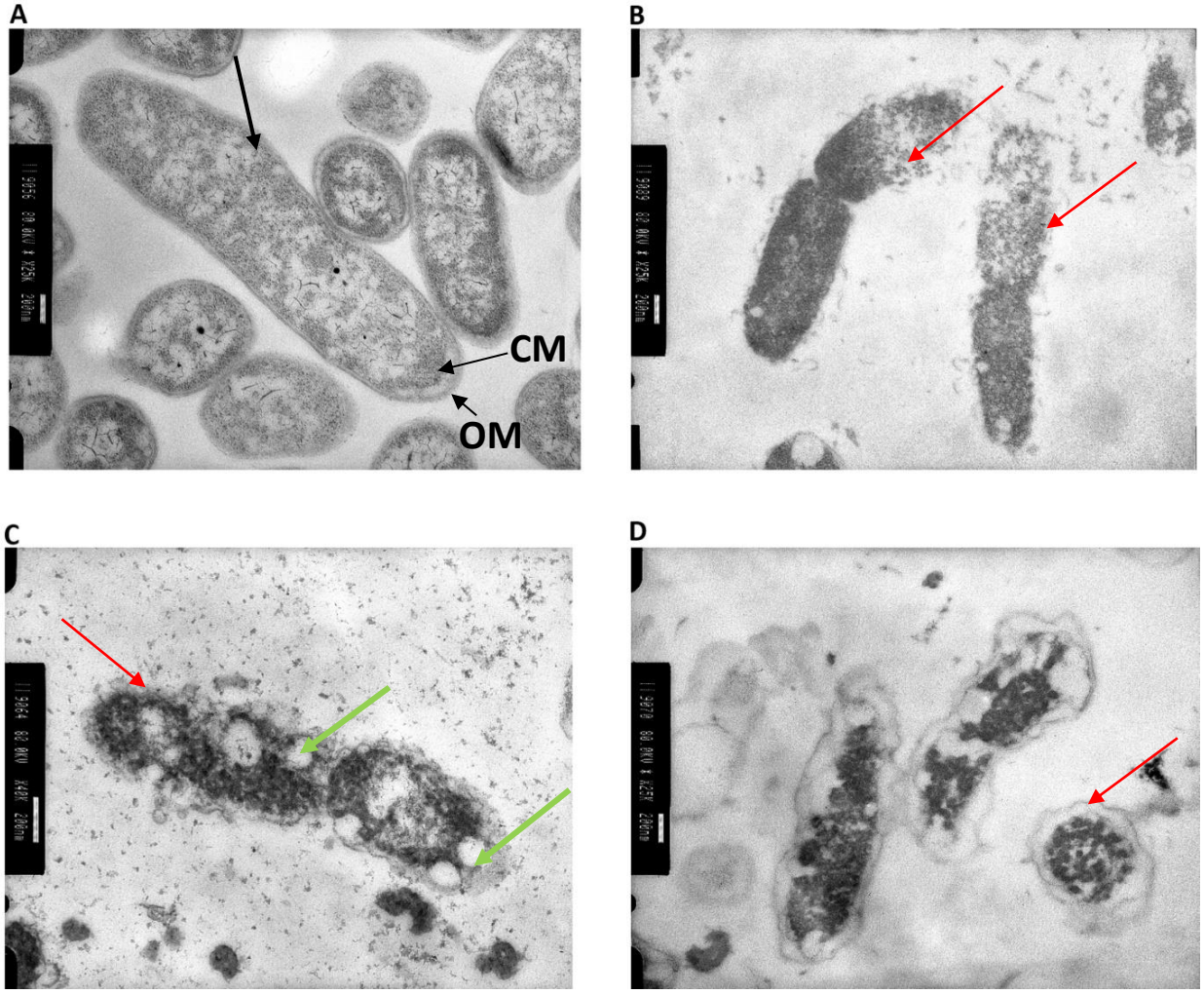


Figure 5.13 (i) TEM images of *E. coli* K12- following 60 minutes (A) Untreated (B) exposure to peptide 2 (C), exposure to peptide 3 (D) exposure to peptide 4, at 3X MIC concentrations. OM = Outer membrane, CM = cytoplasmic membrane Scale bars are 200nm. Magnification is 25,000X (A, B and D) and 40,000X (C).

Transmission electron micrographs of *E. coli* K12 cells revealed that all peptides had varied effects and induced major damage to *E. coli* K12 cell membrane (Figure 5.13). Compared to the untreated cells (5.13 (i) A - black arrow) with intact membrane and intracellular content, large vacuoles (holes) could be observed in cells treated with peptides 5, 7, 8, and 15 (Figure 5.13 (i) - green arrows). Peptides 2, 3 and 4 caused rupturing, and almost a total disintegration of the cell membrane and spilling of intracellular content (Figures 5.13 (i) B, C and D - red arrows).

(ii)

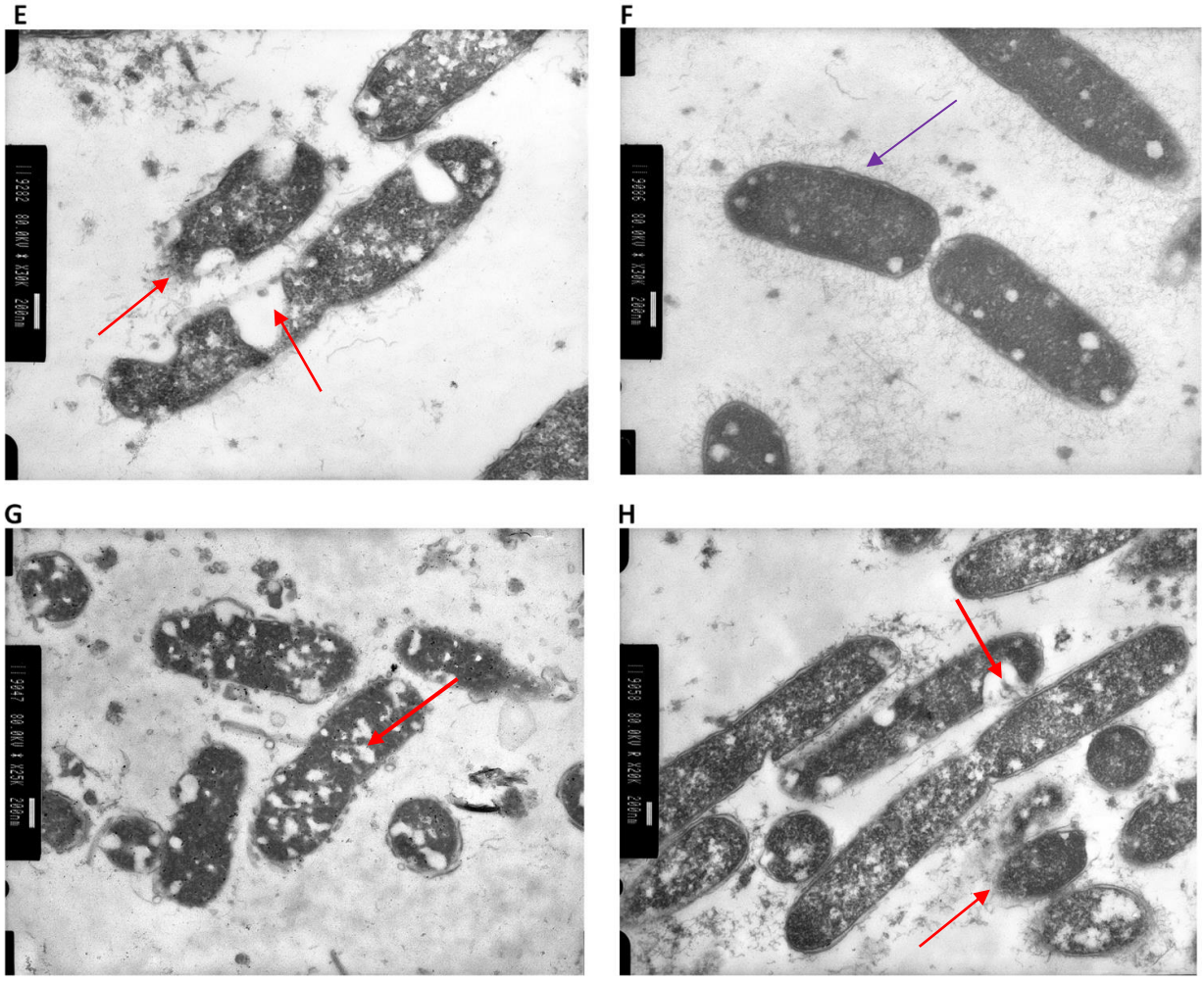


Figure 5.13 (ii) TEM images of *E. coli* K12- following 60 minutes of (E) exposure to peptide 5, (F) peptide 7, (G) exposure to peptide 8 and (H) exposure to peptide 15 at 3 X MIC concentrations. Scale bars are 200nm. Magnification is 30,000X (E and F), 25,000X (G) and 20,000X (H).

Once again, numerous hair-like protrusions and cell shrinkage were observed in cells treated with peptide 7 (Figure 5.13 (ii) F). Blebs on cell membrane surface, vacuoles, as well as a lysed cytoplasm are visible in cells treated with peptides 5, 8 and 15 (Figure 5.13 (ii) E, G and F - red arrows),

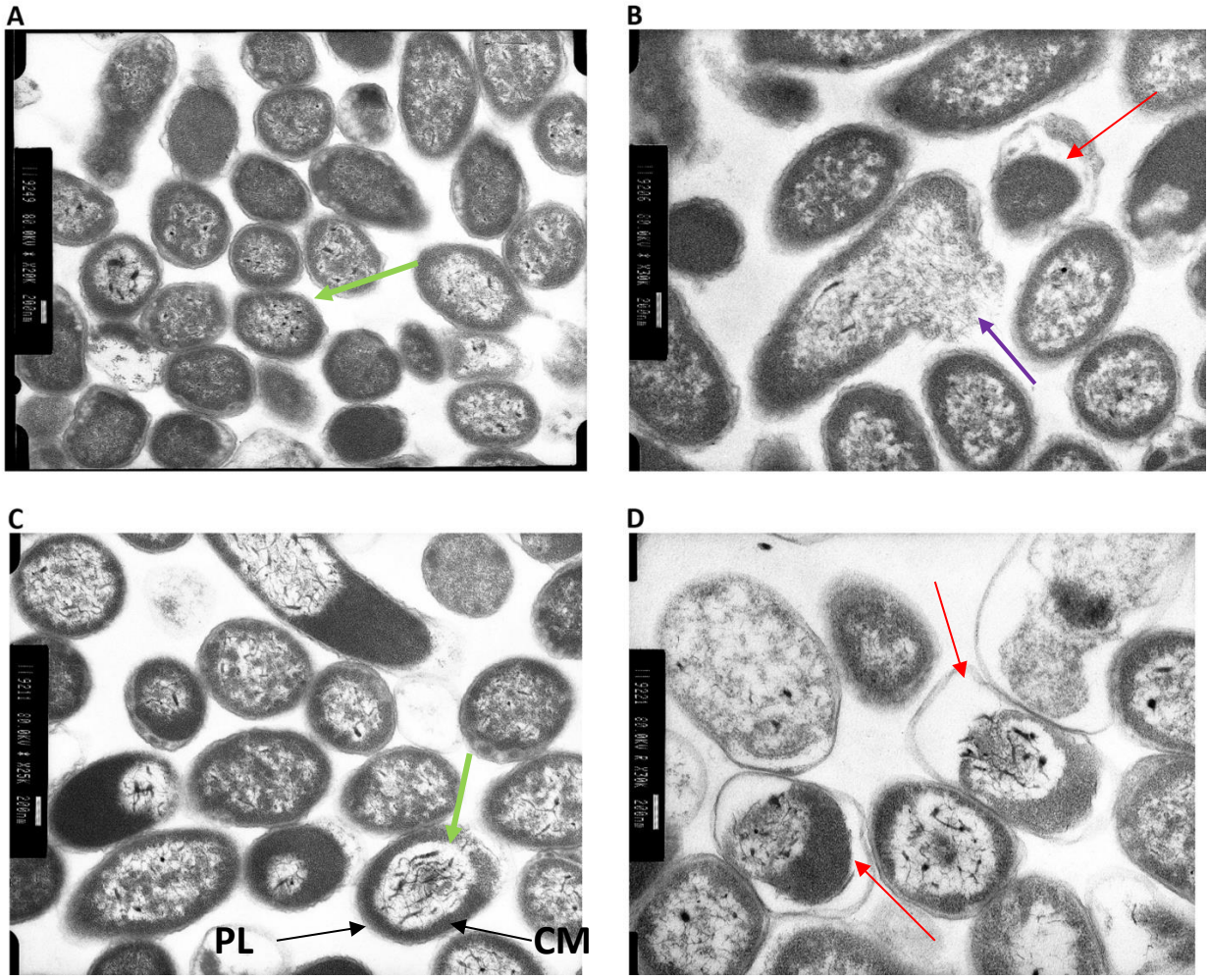


Figure 5.14 TEM images of *Ent. faecalis* - following 60 minutes (A) Untreated (B) exposure to p*alG*1, and after 24 hours (C), untreated (D) exposure *to palG*1 at MIC concentration (200 µg/ml). PL = Peptidoglycan layer, CM = cytoplasmic membrane. Scale bars are 200nm. Magnification is 20,000X (A), 30,000X (B and D) and 25,000X (C).

TEM images of untreated and *palG*1 treated *Ent. faecalis* cells were taken after 1 hour and 24 hours incubation. Untreated cells showed intact oval-spheres (Figure 5.14 A and C - green arrows). However, changes in cell morphology were observed in images of cells treated with *palG*1. Membrane lysis and leakage of cytoplasmic content (Figure

5.14 B - purple arrow), as well as outer membrane detachment (Figure 5.14 B and D - red arrows) in *Ent. faecalis* cells were observed after 1 hour, and more so, after 24 hours of exposure to *palG*1.

5.3.6 Molecular modelling of peptide 3D structures

The structures of peptides 2, 3, 4, 5, 7, 8, 10 and 15 were predicted using PEP-FOLD, while that of *palG*1 was done using Phyre2 server. The three-dimensional structures of the peptides were visualized in PyMOL (Schrödinger 2010). The lowest energy conformation predicted for peptide 2, 3, 4, 5, 7, 8 and *palG*1 were α-helical structures. A β-sheet structure was predicted for peptide 15, Images are shown in Figures 5.15 to 5.22 (a, and b). Table 5.6 below also summarizes some biophysical parameters of the peptides. A periodic chart of amino acids (Figure 5.23) is included in CD/Figshare.

Table 5.6 Biophysical parameters of peptides

peptide	Peptide Sequence	Structure	Length (AAs)	Charge	Total hydrophobicity ratio (%)	Total Arginine and Lysine (R+K) ratio (%)
2	LPRRNRWSKIWKKVVTVFS	α- helix	19	6	42	30
3	HLRRINKLLTRIGLYRHAFG	α- helix	20	5	40	25
4	VLHTGYRKFLHRSKRFFHLR	α- helix	20	6	35	30
5	TMSLRFWRWKVR	α- helix	12	4	33	50
7	NRFTARFRRTPWRLCLQFRQ	α- helix	20	6	40	30
8	AWRWKAFRNCWRVRSSSL	α- helix	18	5	50	27
15	KFVRLKIYCRDKNKGRGISF	β-sheet	20	6	35	35
*palG*1	MRLSHVCSQSSSKYPAGSCN LMTHDRATSSEHRSASRCDR VIRRFCRSDPASRYWRYPVR GMRHGRCHTDW	α- helix	71	+9	29	19

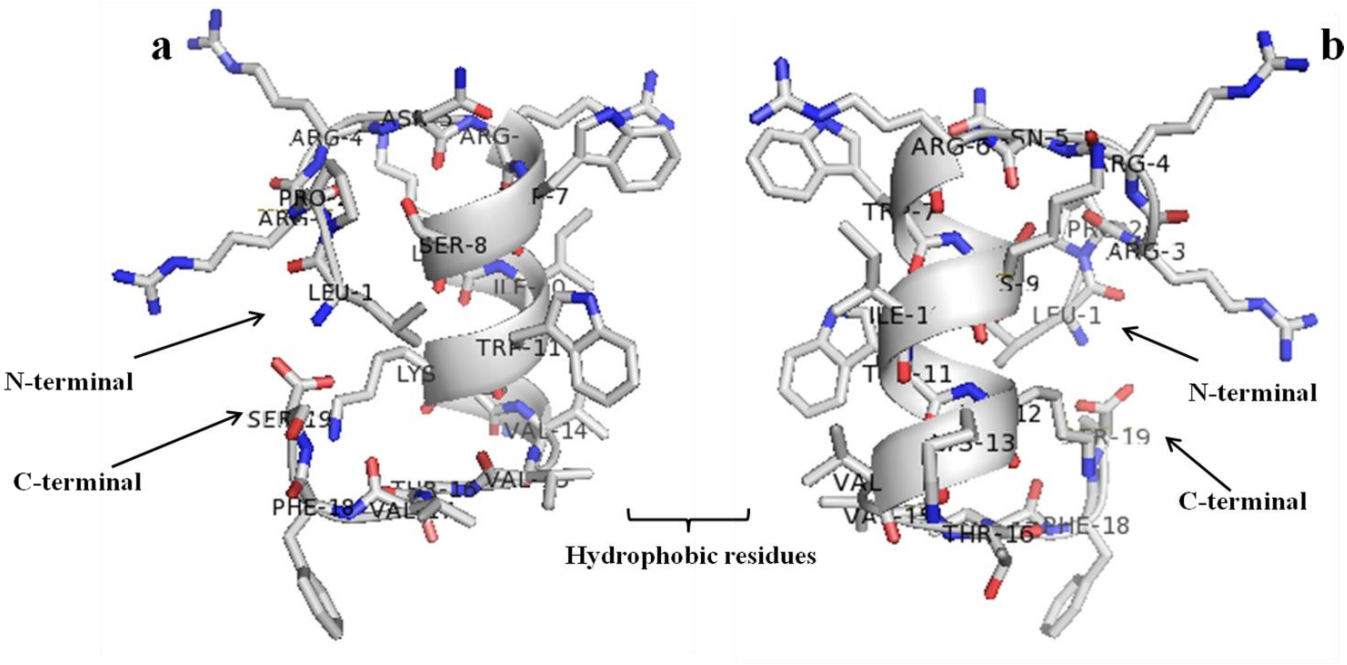


Figure 5.15 Predicted structure of peptide 2 (LPRRNRWSKIWKKVVTVFS). Main-chain and side chains depicted in ribbon and stick representation respectively. Atoms colored according to type: Red: Oxygen; Grey: Carbon; Blue: Nitrogen. Figures were rendered using PyMol.

The hydrophobic residues of the peptide are situated on one side of putative helices, whereas the charged residues are situated on the other, an arrangement typical of many α-helical antimicrobial peptides.

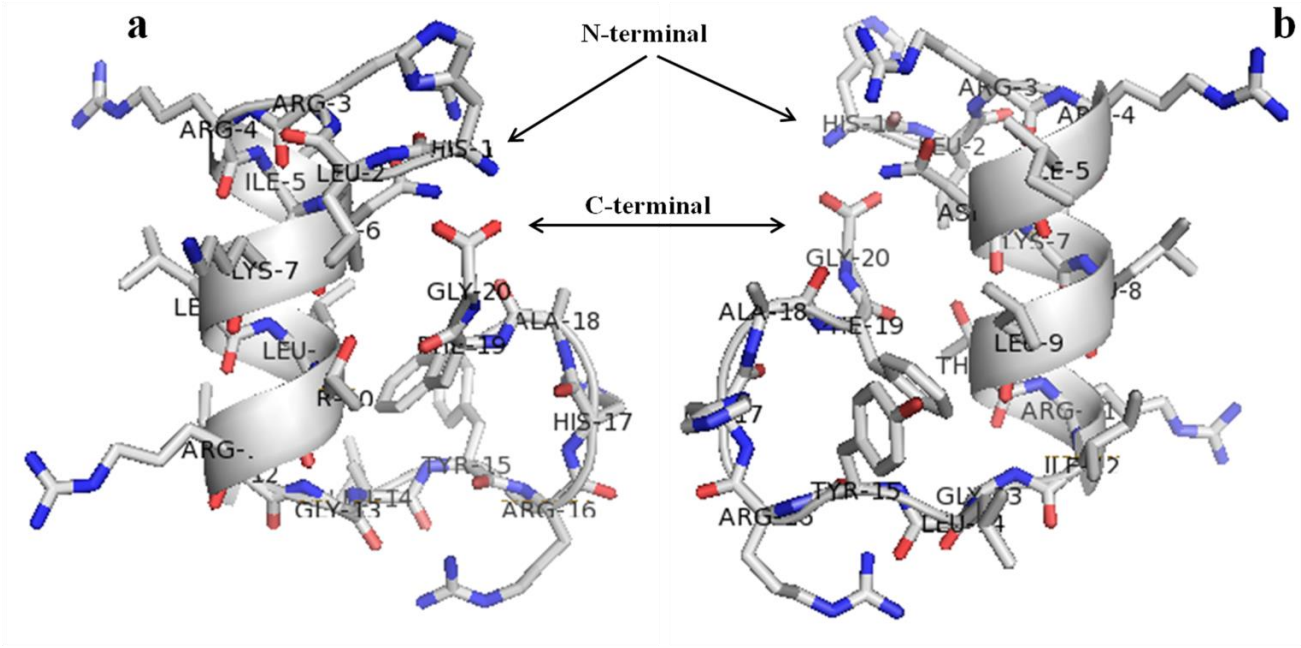


Figure 5.16 Predicted structure of peptide 3 (HLRRINKLLTRIGLYRHAFG). Main-chain and side chains depicted in ribbon and stick representation respectively. Atoms colored according to type: Red: Oxygen; Grey: Carbon; Blue: Nitrogen. Figures were rendered using PyMol.

Peptide 3 has a typical α-helix, again with an amphipathic structure (i.e. hydrophobic and charged residues arranged on either side of the helix).

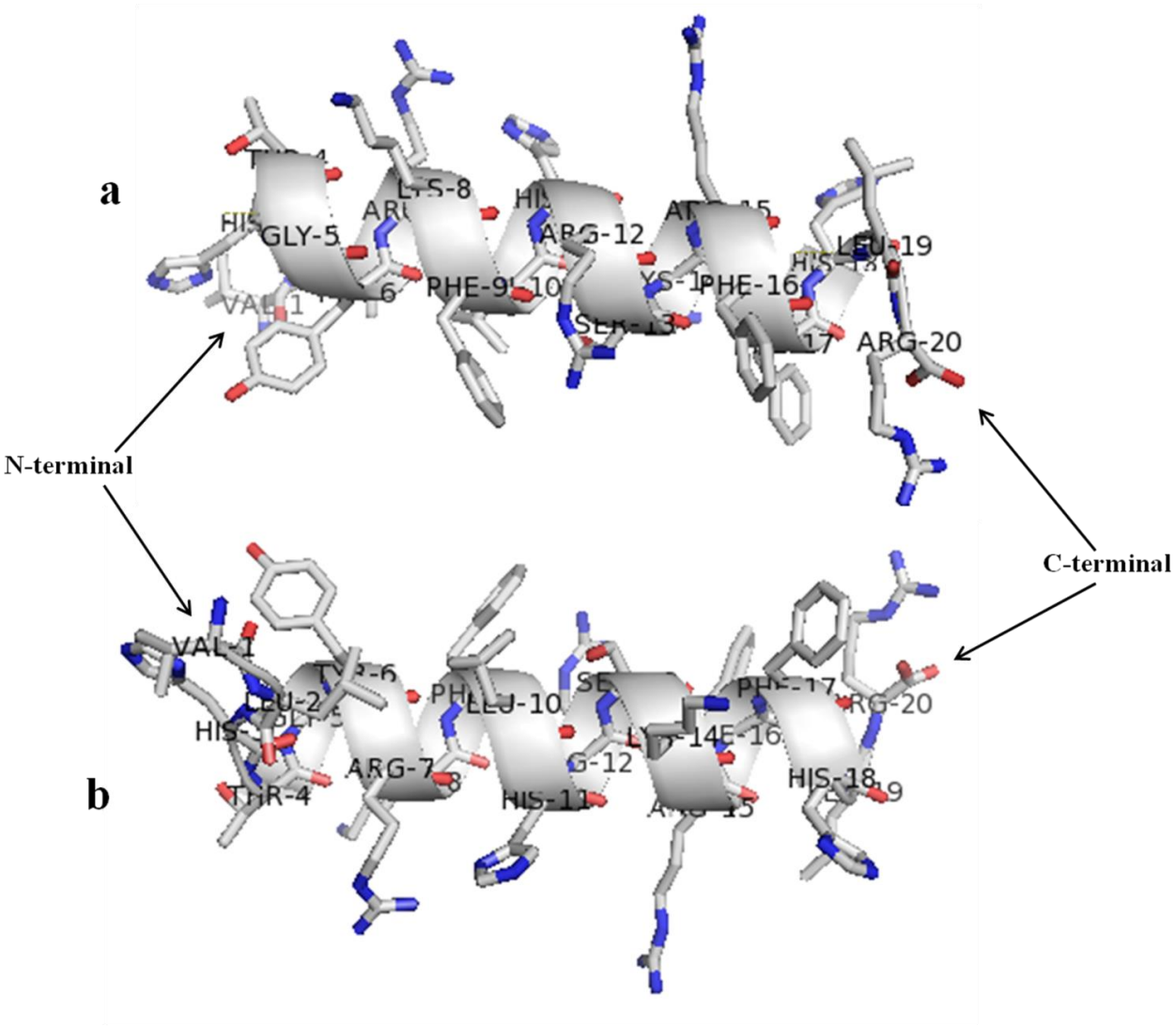


Figure 5.17 Predicted structure of peptide 4 (VLHTGYRKFLHRSKRFFHLR). Main-chain and side chains depicted in ribbon and stick representation respectively. Atoms colored according to type: Red: Oxygen; Grey: Carbon; Blue: Nitrogen. Figures were rendered using PyMol.

Peptide 4 also has a typical α-helical structure, with hydrophobic and charged residues on either side of the helix (hydrophobic residues facing inwards and charged residues outward). In this case, a single α-helix spans almost the entire length of the peptide.

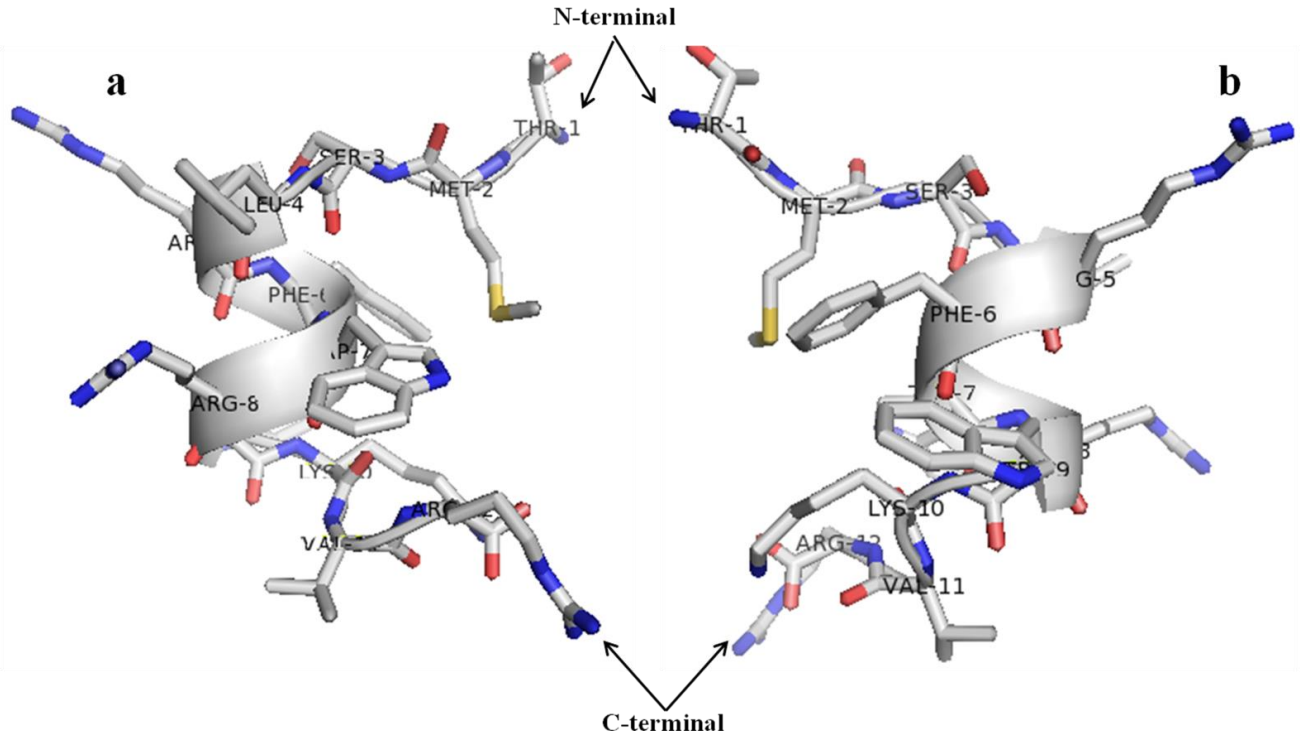


Figure 5.18 Predicted structure of peptide 5 (TMSLRFWRWKVR). Main-chain and side chains depicted in ribbon and stick representation respectively. Atoms colored according to type: Red: Oxygen; Grey: Carbon; Blue: Nitrogen. Figures were rendered using PyMol.

Peptide 5 is a three dent helix, again with hydrophobic residues (e.g. phenylalanine, (position 6) and tryptophan (position 8 and 9)) on one side of the helix, and charged residues (e.g. the C-terminal Arginine (position 12) and lysine (position 10)) on the other side of the helix.

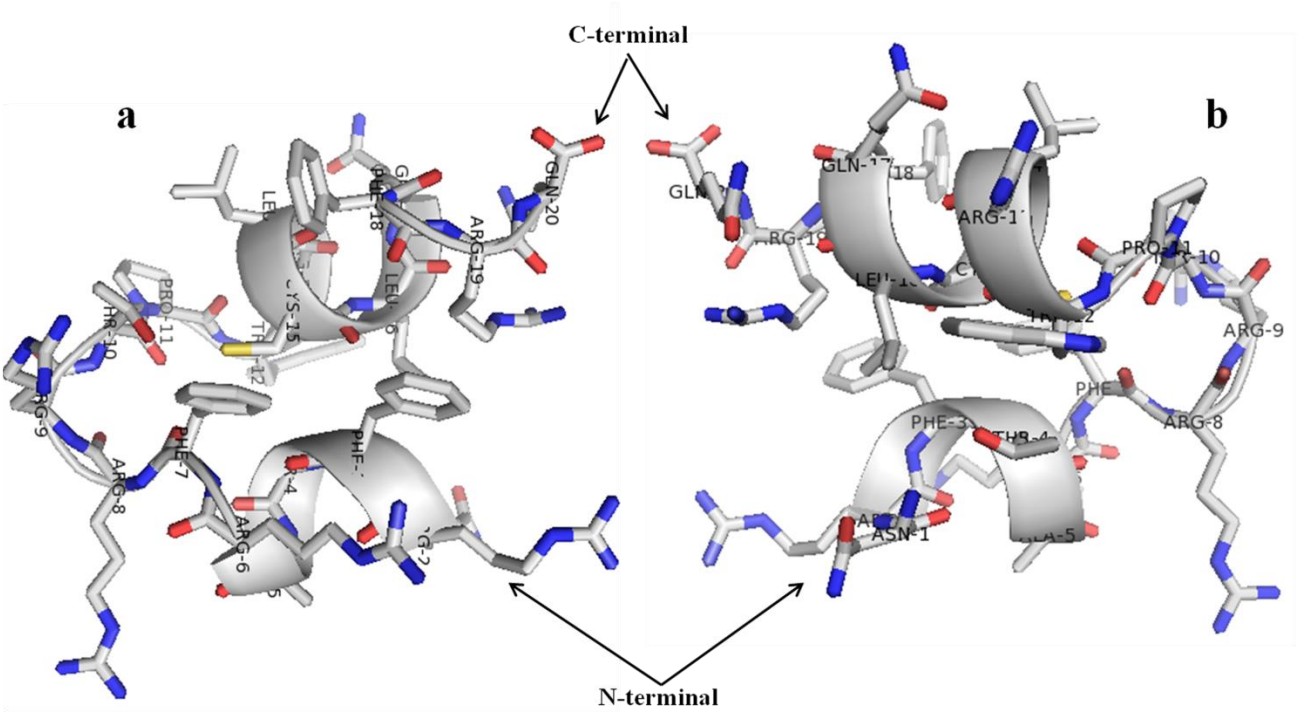


Figure 5.19 Predicted structure of peptide 7 (NRFTARFRRTPWRLCLQFRQ). Main-chain and side chains depicted in ribbon and stick representation respectively. Atoms colored according to type: Red: Oxygen; Grey: Carbon; Blue: Nitrogen. Figures were rendered using PyMol.

Peptide 7 is composed of a pair of antiparallel helices joined by a short turn. The two helices are closely packed with hydrophobic side chains on each helix intercalating in a manner reminiscent of other closely packed helical substructures.

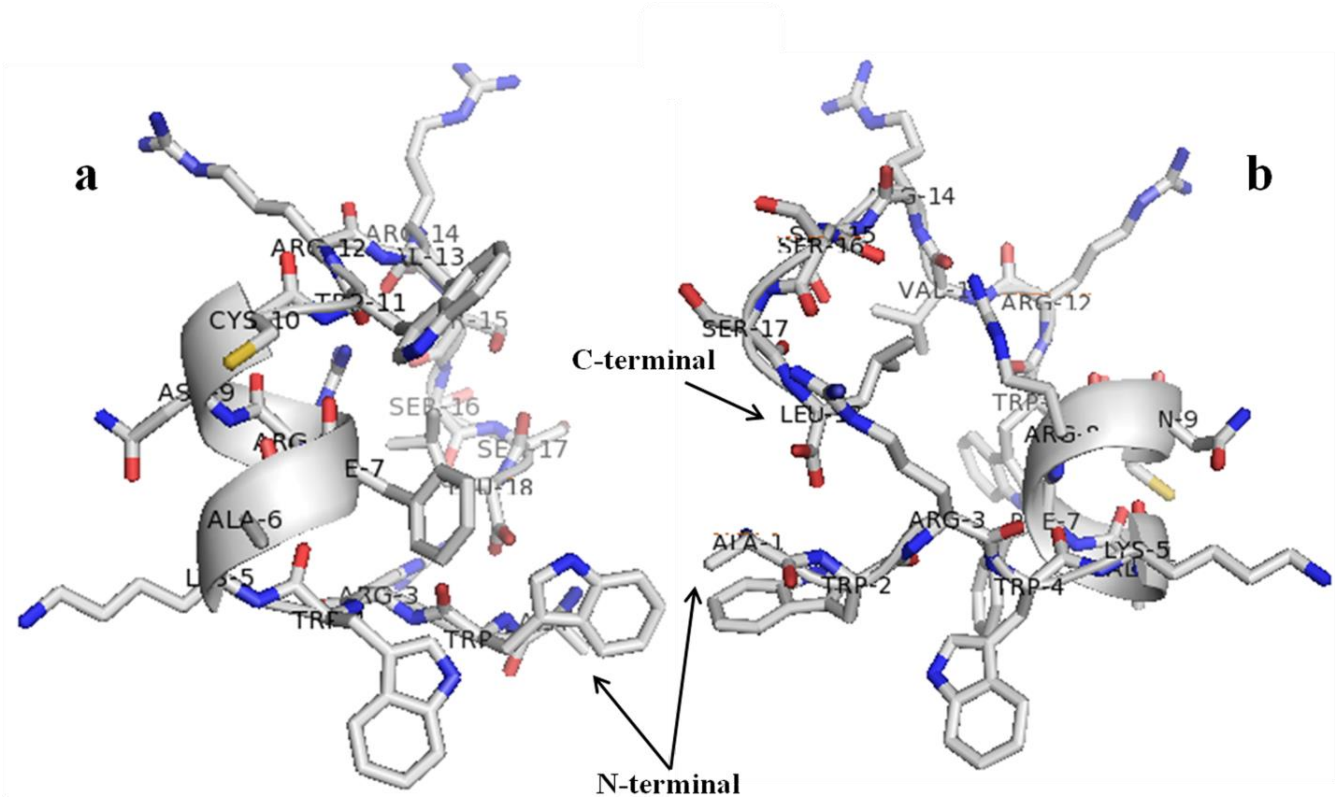


Figure 5.20 Predicted structure of peptide 8 (AWRWKAFRNCWRVRSSSL)**.** Main-chain and side chains depicted in ribbon and stick representation respectively. Atoms colored according to type: Red: Oxygen; Grey: Carbon; Blue: Nitrogen. Figures were rendered using PyMol.

Like peptide 5, peptide 8 is a three dent helix, again with hydrophobic and charged residues either side of the helix.

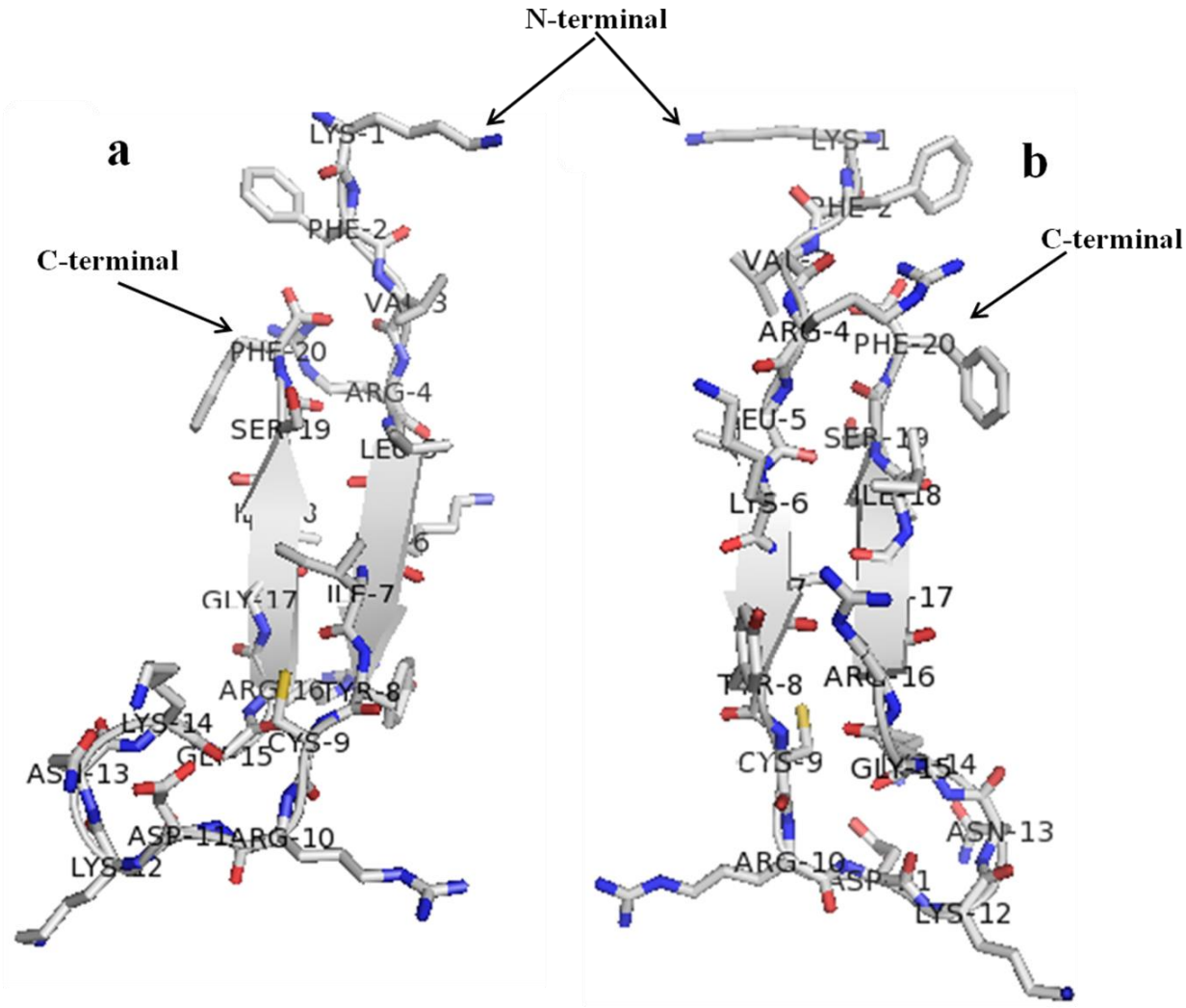


Figure 5.21 Predicted structure of peptide 15 (KFVRLKIYCRDKNKGRGISF). Main-chain and side chains depicted in ribbon and stick representation respectively. Atoms colored according to type: Red: Oxygen; Grey: Carbon; Blue: Nitrogen. Figures were rendered using PyMol.

Peptide 15 takes a typical antiparallel β-sheet turn. This peptide is the only peptide predicted as a β-sheet in this study. Charged residues are again situated on the surface of the peptide (arginine (position 4, 10, 16), aspartic acid (position 11), asparagine (position 13), and Lysine (position 1, 6, 12 and 14)), while hydrophobic residues (e.g valine (position 3, isoleucine (position 7) and phenylalanine (position 2 and 20)) are situated inwards.

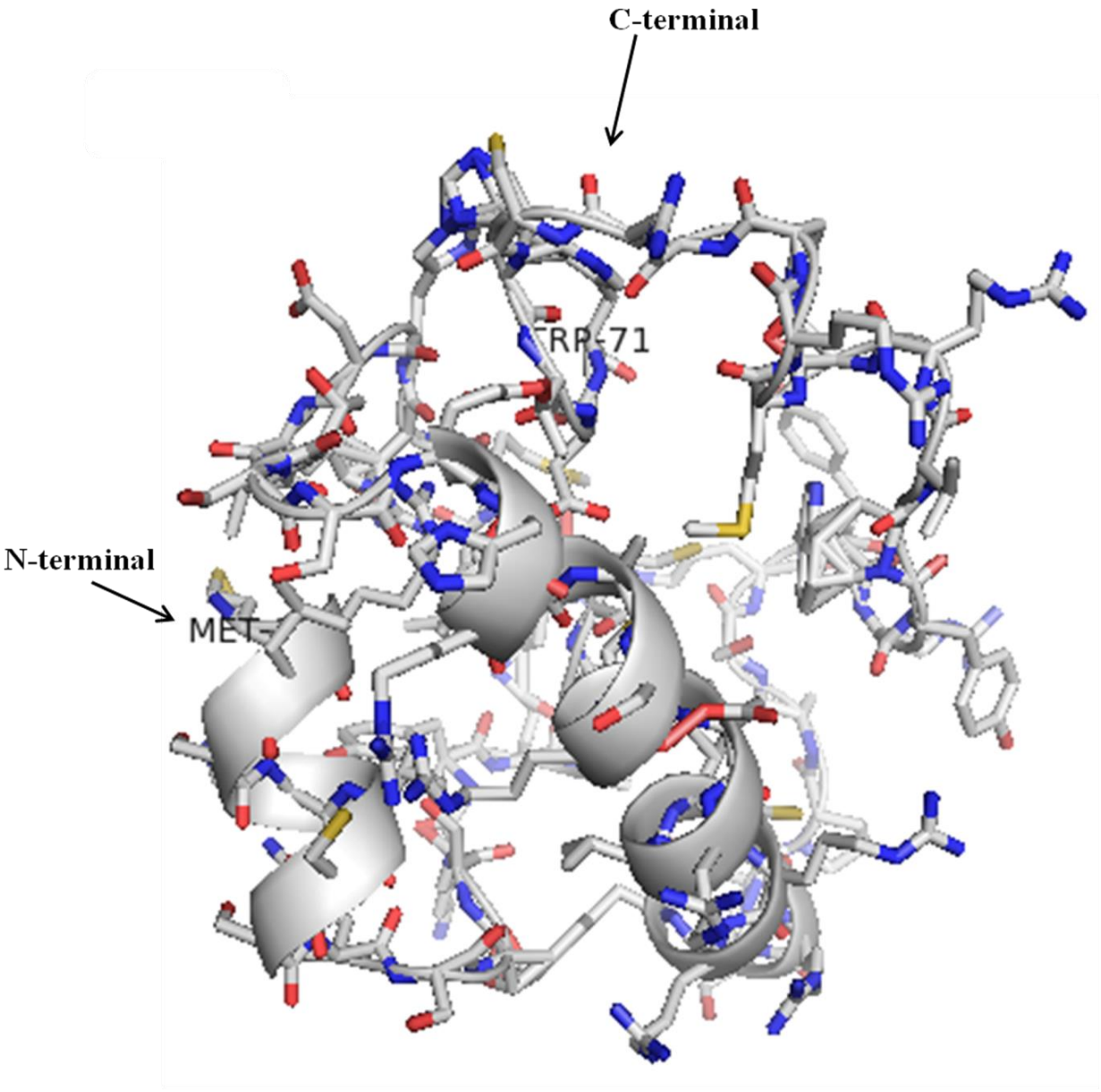


Figure 5.22 Predicted structure of the miniprotein *palG*1 (MRLSHVCSQSSSKYPAGSCNLMTHDRATSSEHRSASRCDRVIRRFCRSDPASR YWRYPVRGMRHGRCHTDW). Main-chain and side chains depicted in ribbon and stick representation respectively. Atoms colored according to type: Red: Oxygen; Grey: Carbon; Blue: Nitrogen. Figures were rendered using PyMol.

The structure of *palG*1 consists of a pair of helices connected by a long turn. It also possesses an amphipathic structure typical of many AMPs.

5.4 Discussion

Many of the synthesized peptides had similar activities against all microorganisms with an average MIC of 32 µg/ml. Peptides, 2, 3, 4, 5 7, 8, 10 and 15 had a broader activity spectrum than all other peptides tested. Some peptides which had activity in the peptide library screen (6, 11, 12, 13, 14) showed no activity when they were synthesized on resin. This may be caused by the difference in method of synthesis or folding of the peptide. Peptide 12 (QVRWWGRYWRRKWATCR) for instance, has an N-terminal Glutamine, which might form glutamic acid and inactivate the peptide. Therefore, the peptide was resynthesized with an acetylated N-terminus and retested for activity against EMRSA-15 (new MIC 32 µg/ml). *palG*1 was only active against *Ent. faecalis* with an MIC of 200 µg/ml. It also showed slight inhibition of *E. coli* K12 growth (observed in growth curves) with no detectable MIC at the highest concentration tested. Although, *palG*1 was successfully expressed in *E. coli* TOP10 cells, this mild inhibition observed here, may account for the low level of expression and yield of the protein.

Time kill studies demonstrated time dependent microbicidal action of peptides 2, 3 and 7 against EMRSA-15 and peptides 2, 3, 4, 5, 7, 8 and 15 for *E. coli* K12 in MH broth, a rich medium. After exposure to peptides, reductions of >8 $\log_{10}$ CFU/ml (peptides 2, 7 after 1 and 3 hours respectively) and >3log10 CFU/ml (peptide 3 after 30 minutes) of EMRSA-15 cells were demonstrated. Reductions of >8 $\log_{10}$ CFU/ml of *E. coli* K12 cells was observed for all peptide treatments. Apart from peptide 4, where *E. coli* K12 cells seemed to recover after 4 hours, no increase in cell density or cell numbers was observed for all other peptide upon continued incubation (up to 24 hours (results not shown) indicating possibly a complete kill. *palG*1 demonstrated over 70% kill against *Ent. faecalis* cells over a 24 hour period. Also, for all pathogens tested, no antimicrobial

activity was observed in peptide free controls, demonstrating that antimicrobial activity was directly related to the addition of the peptides to the bacteria cultures.

There was no obvious correlation between antimicrobial activity and membrane depolarisation for many of the short AMPs, a phenomenon that has previously been observed in CP29 and Bac2A-NH2 (Wu and Hancock 1999). Individual peptides demonstrated varied membrane depolarising ability. Apart from peptide 8, which had >50% membrane depolarisation of *E. coli* K12 cells, other peptides had moderate membrane depolarisation effects (10-30% depolarisation) against both *E. coli* K12 and EMRSA-15. This may be because other factors, and not membrane depolarisation alone are responsible for killing of cells. No membrane depolarisation was observed in *Ent. faecalis* cells treated with *palG*1. Irrespective, the greater permeabilization and overall faster killing kinetics of *E. coli* K12 with respect to EMRSA-15, and its susceptibility to the various peptides, confirm the conclusion from previous studies that the double membrane of the Gram-negative bacterium is a less efficient barrier than the single membrane and peptidoglycan layer of the Gram-positive bacterium (Tossi et al. 1997, Tiozzo et al. 1998).

Transmission electron micrographs revealed that compared to untreated cells, which retained their morphology, all peptide-treated cells had distorted cell morphology. This is evidence that the peptides may interact with the cell division machinery of the cells. TEM images of all untreated bacteria cells showed smooth, intact surfaces while peptide treated cells showed breakage and roughness of the cell wall in general. Peptide 3 induced outer cell detachment and spilling off of the cytoplasmic content in EMRSA-15. Moreover, the cell wall appeared to be severely damaged and showed undulations indicating a loss of integrity. EMRSA-15 cells treated with peptides 2 and 7 had markedly reduced sizes (cell shrinkage) compared with the untreated cells. This cell

shrinkage may be due to loss of turgor (Pathak and Chauhan 2011) or may represent a type of stress response to the peptides or that cells are going into dormancy. Mesosome-like structures were also observed in EMRSA-15 cells treated with peptides 2 and 3. These mesosomes are intracytoplasmic membrane inclusions which have been confirmed as a definite pattern of membrane organisation in *S. aureus* cells treated with antibiotics, including antimicrobial peptides (Balkwill and Stevens 1980, Friedrich et al. 2000, Santhana Raj et al. 2007, Rabanal et al. 2015). Their presence indicates cytoplasmic membrane alteration/damage and possible uncoupling of the synthesis of call wall polymers caused by antimicrobial activity (Friedrich et al. 2000). Compared to the untreated *E. coli* K12 cells which had intact membranes and intracellular content, large vacuoles (holes) could be observed in cells treated with peptides 5, 7, 8, and 15. Peptides 2, 3 and 4 seemed to cause rupturing and almost a total disintegration of the cell membrane and spilling of intracellular content. In addition, a compete loss of membrane integrity can be seen in some of the peptide treatments, with some showing 'empty nests' and missing cytoplasm. A type of cell shrinkage is again observed in *E. coli* K12 cells treated with peptide 7. Membrane lysis and leakage of cytoplasmic content, as well as, outer membrane detachment were observed in *Ent. faecalis* cells treated with *palG*1 for 1 hour, and more so, after 24 hours.

The hairy appearance observed in some of the peptide treated cells under TEM could be due to an electrostatic interaction between the peptides and the cell surface. This phenomenon has been observed in another study (Rojas-Chapana et al. 2005) and can be explained through the interaction between the positively charged surface of the peptides and the negatively charged bacterial cells. This observation is also in good agreement with the structural models predicted for these peptides. Structural modelling of peptides revealed that all peptides had α-helical conformations apart from peptide 15,

which is a β-sheet. The hydrophilic-hydrophobic behaviour of these peptides as evident in the predicted peptide structures (hydrophilic/charged residues on one side, and hydrophobic residues on the other side), and their interaction with phospholipids, could also play a role in the penetration and perforation of the bacterial surface. All peptides have a net positive charge ≥4, which attracts them to the anionic microbial membranes and an ability to assume amphipathic structures (hydrophobicity ratio of >30%) that favour insertion into microbial membranes (Hancock and Lehrer 1998). There is evidence in many studies that computer modelling studies correlate with structures indicated by circular dichroism (CD) -an excellent method of determining the secondary structure of proteins (Greenfield 2006). However, this is not always the case and CD studies in different solutions/environments, as well as, X-ray crystallography and Nuclear magnetic resonance (NMR) methods, which give the residue-specific information (Greenfield 2006) may be required to confirm the accuracy of predicted structures upon peptide interaction with membranes.

As no peptide was haemolytic to sheep erythrocytes up to concentration of 500 μg/ml, activity of these peptides may be prokaryotic specific suggesting their use in intravenous treatment applications in mammals. It is however, necessary to carry out cytotoxicity assays on human and other mammalian cell lines to determine whether the peptides can induce necrosis in cells. In addition, although AMPs are known to possess remarkable resistance to microorganism adaptation, the mutation frequencies to ascertain the likelihood of resistance arising in the use of these peptides were not determined in this study.

In conclusion, *in vitro* susceptibility data suggests that whereas all synthesized AMPs had similar activity against *E. coli* K12, peptides 2, 3, and 7 have the most activity against EMRSA-15. Loss of viability also seemed to have occurred in the first hour and

perhaps simultaneously as membrane depolarisation and/or damage. Data presented here support the potential use of AMPs (2, 3, 7), AMPs (2, 3, 4, 5, 7, 8, 15) and the polypeptide *palG*1 in the treatment of *S. aureus*, *E. coli* and *Ent. faecalis* infections respectively in the future. We are not aware of other reports of isolation of clinically relevant antimicrobial peptides from cow rumen using metagenomics methods. These AMPs show activity against human pathogens and have potential application in the treatment of multidrug resistant pathogens. Furthermore, this novel antimicrobial peptides could provide templates for the design of further novel therapeutics in the future.

CHAPTER SIX

General discussion, conclusions and future work

6.1 General discussion

Bacterial resistance to classical antibiotics poses a growing challenge to medicine and public health. Increasing resistance to preferred therapies has limited the options for treatment, and as a result, the scientific community is urged to develop new drugs in order to efficiently control resistant microorganisms (Lipsitch 2001). These new antimicrobial compounds must have potent antimicrobial activity but without undesirable side effects and with low risk of microbial resistance arising (Perry and Hall 2009). Among these new compounds, antimicrobial peptides (AMPs) and derivatives have emerged as an important field of research in the fight of microbial resistance (Yeaman and Yount 2003, Toke 2005, Guani-Guerra et al. 2010). Antimicrobial peptides (AMPs) are a growing class of natural and synthetic peptides with a wide range of targets including viruses, bacteria, fungi and parasites (Brown and Hancock 2006). Their ability to avoid bacterial resistance mechanisms due to their multiple mechanism of action, broad range of activity and low/selective toxicity to hosts (Yeaman and Yount 2003, Matsuzaki 2009) makes them particularly interesting as potential lead compounds.

Most antibiotics introduced into the clinic were discovered by screening cultivable soil microorganisms (Ling et al. 2015). More recently, the marine environment has also been recognised as a large potential source of biodiversity and it is being increasingly searched for novel chemicals with useful bioactivity (Blunt et al. 2007, Simmons et al. 2008, Liu et al. 2010, Hu et al. 2011). However, as natural product resources are practically inexhaustible, the majority of the world's biodiversity remains to be explored (Harvey 2007, Berdy 2012) as approximately 99% of all species in external

environments do not grow under laboratory conditions (Lewis 2013). Several studies already suggest that new organisms such as uncultured bacteria are likely to harbour new antimicrobials (Degen et al. 2014, Doroghazi et al. 2014, Gavrish et al. 2014, Wilson et al. 2014). The metagenomes of complex microbial communities are also rich sources of novel biocatalysts (Kang et al. 2015). Therefore, exploiting uncultured bacteria in these different natural and complex environments is likely to revive the platform of natural product/drug discovery (Lewis 2012). The rumen microbiome, which has also been cited as a resource of novel enzymes especially for cellulosic biofuel production (Rubin 2008, Hess et al. 2011) is one of the many unexploited environments for novel antimicrobial compound discovery. It presents a rich and underutilized source of novel enzymes with tremendous potential for industrial application. Advent of nucleic acid-based molecular technologies has ushered in a new culture-independent perspective of microbial ecology unbiased by the culturing aptitude of microbial species (McCann et al. 2014). Among the methods designed to gain access to the physiology and genetics of uncultured organisms, metagenomics, the genomic analysis of a population of microorganisms, has emerged as a powerful centrepiece (Handelsman 2004). The potential for application of metagenomics to biotechnology seems endless as functional screens have identified new enzymes, antibiotics and other reagents in libraries from diverse environments (Gillespie et al. 2002, Lorenz and Schleper 2002, Piel 2002, Voget et al. 2003).

The aim of this thesis was to characterise antimicrobial proteins and peptides (AMPPs) from the rumen microbiome using metagenomic approaches and bioinformatic tools. In order to do this, a number of microbiology, molecular and computational/informatics methods were used. Firstly, a fosmid-based cow rumen metagenomic clone library created from the solid attached bacteria (SAB) of rumen content was functionally

screened for antimicrobial activity (Chapter 3). Clones with antimicrobial activity were subsequently sequenced after which antimicrobial genes were expressed (genes/proteins ≥25 AAs in length) or chemically synthesized (<25 AAs in lenghth) (Chapter 4), and characterised for antimicrobial activity (Chapter 5). Secondly, sequence data from an already existing cow rumen metagenomic dataset was bioinformatically mined for antimicrobial proteins and peptide sequences (Chapter 3), which were then characterized for antimicrobial activity against bacterial pathogens in the same way as the antimicrobial genes identified from the IBERS fosmid metagenomic library (Chapter 4 and 5) in order to gain understanding of their applicability in the treatment of infections.

Functional and sequence based metagenomic screening strategies were used complementarily as they both present advantages and limitations (Uchiyama and Miyazaki 2009). Whereas, sequence homology based analysis allows for identification of new enzymes from a range of environments, discovery is limited to the identification of members from known gene families. Functional screening of metagenomic libraries on the other hand does not depend on previous knowledge and so has the potential to discover novel classes of genes coding known or new functions (Ferrer et al. 2009, Simon and Daniel 2009).

In the present study, novel antimicrobial genes were identified from the fosmid based cow rumen metagenome library (Prive 2011) and from an already existing cow rumen metagenome (Hess et al. 2011). This is no surprise as AMPs are components of the innate host immune/defence system found among all classes of life ranging from prokaryotes to humans (Boman 1995, Zasloff 2002, Giuliani et al. 2007). Five longer antimicrobial genes/proteins (Gene 6, 17A, 17B *palG*1 and *palG*2) were identified from the in-house metagenome library, including 181 short antimicrobial peptides. Six genes

H-G1, H-G2, H-G3, H-G4, H-G5, and H-G6 were identified from the (Hess et al. 2011) dataset. Antimicrobial proteins- longer genes were amplified and expressed in a suitable *E. coli* host while shorter genes (≤22 AAs 135 in total) were chemically synthesized in a peptide library.

After initial antimicrobial and haemolytic activity screens, seven (7) of the short chemically synthesized AMPs designated peptides 2, 3, 4, 5, 7, 8 and 15, as well as, *palG*1, the only longer protein successfully expressed and purified, were chosen for further analysis. These AMPPs displayed antimicrobial activity (around 32 to 64 µg/ml) against both Gram positive bacteria strains (including Methicillin sensitive and resistant *S. aureus* strains MSSA RN4220 and EMRSA-15, *Ent. faecalis* JH2-2 and *List. monocytogenes* NCTC 11994 (serovar 4b)), as well as, Gram negative bacteria strains (*E. coli* K12, *Salmonella enterica* serovar Typhimurium SL1344 and *P. aeruginosa* (15692) PAO1 strain H103) in MHB. These MIC values fall in the range of data from published work for other cationic AMPs (Zasloff 1987). The MIC of *palG*1 against *Ent. faecalis* was 200 µg/ml. Time kill studies carried out at 3 X MIC demonstrated time-depended microbicidal action of peptides against EMRSA-15 and *E. coli* K12 alike. Reductions of >8 $\log_{10}$ CFU/ml (peptides 2 and 7 after 1 and 3 hours respectively) and >3 log10 CFU/ml (peptide 3 after 30 minutes) of EMRSA-15 cells were demonstrated. Reductions of >8 $\log_{10}$ CFU/ml of *E. coli* K12 cells was observed for all peptide treatments. Apart from peptide 4 where *E. coli* K12 cells seemed to recover after 4 hours, no increase in cell density or cell numbers was observed for all other peptides upon continued incubation (up to 24 hours (results not shown) indicating a possible bacterioststic effect. *palG*1 demonstrated over 70% kill against *Ent. faecelis* cells over a 24 hour period. This rapid kill within minutes, observed with the peptides in this study is also a common observation with many AMPs (Maloy and Kari 1995, Hancock and

Lehrer 1998, Shai 2002). All peptides were non-haemolytic against sheep erythrocytes and only around 10% haemolysis was observed even at concentrations up to 500 µg/ml. This suggests that the peptides may have selective activity against microbial cells, thus making them promising candidates for alternative therapies, not just in topical applications but potentially in intravenous use against MDR bacteria. It is however, necessary to carry out cytotoxicity assays on human and other mammalian cell lines to determine whether the peptides can induce apoptosis and necrosis in cells (Paredes-Gamero et al. 2012).

The peptides depolarization capacity was tested by diSC_3(5) method for EMRSA-15 and *E. coli* K12, a Gram-negative and Gram-positive strain respectively. An increase in the fluorescent signal, albeit moderate (between 10-50% membrane depolarisation) compared to the Triton-X 100 treatment was observed shortly after the addition of the short AMPs, indicating that peptides are active against both organism types. No membrane depolarisation was observed in *Ent. faecalis* cells treated with the longer AMP, *palG*1. This may be because *palG*1 may require a longer time (>20 minutes) to induce membrane depolarisation (as is observed in the time kill assay). Irrespective, the depolarization capacity is higher in *E. coli* K12, the Gram-negative strain tested, probably because of the easier access of peptides to the cytoplasmic membrane. This confirms the findings from previous studies that the double membrane of Gram-negative bacterium is a less efficient barrier than the single membrane and peptidoglycan layer of the Gram-positive bacterium (Tossi et al. 1997, Tiozzo et al. 1998). Moreover, as an outer-membrane-barrier defective mutant *E. coli* strain was not used as required for some antimicrobial agents (Wu and Hancock 1999, Zhang et al. 2000), it can be assumed that peptides by themselves can destabilize *E. coli* K12 outer membrane and have an effect on cytoplasmic membrane, without requiring any

additional treatment to remove the outer layer barrier. This could be a result of peptide interaction and binding to lipopolysaccharides (LPS) or peptidoglycans (PGNs) for *E. coli* K12 and EMRSA-15 respectively (Torrent et al. 2008) and would need to be confirmed using the appropriate assays. PGNs are a major constituent of Gram-positive bacteria cell walls, which can induce several inflammatory responses upon bacterial infection (Dziarski and Gupta 2005). It is believed that peptide binding to PGNs at the bacteria surface can trigger the cell autolysins (Sahl et al. 2005), thus leading to cell death. Given the low depolarising activity of the short AMPs in this study, it would seem that membrane-destabilizing activity does not solely explain the bactericidal capacity of these peptides.

Furthermore, TEM images of the peptide treated cells also confirmed cell wall and inner membrane damage leading to large vacuoles in the cytoplasm, cell rupture, shrinkage and disintegration in some cases. This provides additional evidence of a putative multimodal action of the peptides as previously described in other AMP studies (Pathak and Chauhan 2011, Parween et al. 2013, Rao et al. 2013). In *E. coli* K12 for instance, membrane depolarisation observed in the peptides (especially peptide 8) and the concomitant physical destabilization of the outer membrane as observed in the TEM images is by itself an antimicrobial mechanism.

The antimicrobial peptides tested in this study are mostly cationic α-helical peptides with the exception of peptide 15 which has a β-sheet structure as predicted by computer modeling using peptide sequences. Their structures display a cationic and amphiphilic nature which complies with the main "rules" for antimicrobial peptide definition (Hancock and Sahl 2006). They have an overall positive charge and contain a good proportion of hydrophobic residues. This positive charge should greatly facilitate their accumulation at the polyanionic microbial cell surfaces. Many AMPs bind in a

membrane parallel orientation, interacting only with one face of the bilayer and this may be sufficient for antimicrobial action (Hwang and Vogel 1998), thus perturbing the membrane integrity. Some peptides and phospholipids have been shown to translocate or form multimeric transmembrane channels promoting the membrane depolarization that also seem to contribute to their activity (Shai 1999, Bhattacharjya and Ramamoorthy 2009) at higher concentrations. The amphipathic nature of the predicted peptide structures in this study as well as the membrane damage observed in the TEM images is in good agreement with this type of interaction. TEM images indicate that the peptides are membrane destructive and although membrane disruptive peptides have been reported to be of the α-helical structural class (as is the case for most of the peptides in this study), not all α-helical peptides are membrane disruptive (Park et al. 1998, Friedrich et al. 2001, Patrzykat et al. 2002). Even peptides with β-sheet structure (as in the case of peptide 15 in this study- Figure 5.21) can be membrane disruptive. For example, gramicidin, a highly hydrophobic β-sheet peptide with a lethal mechanism of action has been shown to be involved in cytoplasmic membrane permeabilization and form disruptive transmembrane pores and cation specific channels (Burkhart et al. 1998, Panchal et al. 2002). Still it is difficult to say which of the known membrane interaction and disruption models (i.e. barrel stave, carpet models, or micellar aggregate model) explains the activity of these peptides without further experimental evidence.

6.2 Conclusions

Antimicrobial peptides are promising candidates for overcoming the critical and accelerating problem of bacterial resistance to currently utilized antibiotics. The high cost and low yield of isolation from natural sources, which hindered peptide application in medicine in the past have been overcome by new technological advances and improvements in traditional recombinant methods (Ling et al. 2015). Using a

combination of metagenomic and bioinformatic techniques in this project, the rumen has been shown to be a great resource for bio-prospecting of novel antimicrobial proteins and peptides. AMPs discovered in this study display low haemolytic activity against blood cells and have broad-spectrum activity against both Gram positive and Gram negative organisms alike, including epidemic methicillin resistant *S. aureus* EMRSA-15, *E. coli* K12 and *Ent. faecalis*. The results presented here indicate events that lead to loss of cell viability to be cytoplasmic leakage and some evidence of interference with the cell division mechanism as observed in TEM images. The non-haemolytic property of the peptides suggest that they could be used both as topical agents and intravenously in the treatment of MDR bacterial infections. Overall, the data support the potential use of AMPs (2, 3, 7), AMPs (2, 3, 4, 5, 7, 8, 15) and the polypeptide *palG*1 in the treatment of *S. aureus*, *E. coli* and *Ent. faecalis* infections respectively in the future. To the best of my knowledge, this is the first study to prospect the cow rumen metagenome for novel antimicrobials. The antimicrobial compounds discovered here are not only novel, but demonstrate potent activity against clinically relevant human pathogens, indicating that the rumen is an untapped goldmine for the discovery of antimicrobials. These AMPPs could have direct application either in the treatment of infections caused by these pathogens or form templates for the development of new drug targets in the future.

6.3 Future work

Due to time constraints, alternative gene cloning and protein expression methods could not be explored especially for those antimicrobial proteins which failed to express *in vivo* in *E. coli* using the pTrcHis TOPO expression system. It would also be necessary to optimize *in vitro* expression of proteins in order to recover expressed protein in high enough yields for downstream applications.

Since the viable count and optical density (OD) methods used in this study are not a real time measure of viability, the activity of macromolecular biosynthetic pathways following exposure to peptides should be considered. Further mechanism of action studies using advanced bio-imaging technologies, like confocal microscopy etc with radio-labelled peptides as well as ionic leakage assays would also be required to completely understand the killing mechanism of these peptides. It is also necessary to perform cytotoxicity assays on human and other mammalian cell lines to determine whether the peptides can induce necrosis in cells. Circular dichroism studies in different solutions/environments as well as X-ray crystallography and Nuclear magnetic resonance (NMR) methods, which give the residue-specific information (Greenfield 2006) may be required to confirm the accuracy of predicted structures upon peptide interaction with membranes.

Furthermore, although AMPs such as the ones identified in this study, are known to possess remarkable resistance to microorganism adaptation, the mutation frequencies to ascertain the likelihood of resistance arising in the use of these peptides were not determined in this study and need to be determined in the future. Moreover, as many AMPs are known to prevent biofilm formation or eradicate already established biofilms (Batoni et al. 2011, Jorge et al. 2012, Di Luca et al. 2014), it will be useful to investigate the effect of the AMPs discovered in this study on biofilm populations. An open-access, manually curated database of AMPs specifically assayed against microbial biofilms (BaAMPs, accessible at http:// www.baamps.it) has recently been generated and will benefit AMP-biofilm research and support the design of novel molecules active against biofilms (Di Luca et al. 2015). In the long run, the efficacy of these AMPs in animal models would be tested.

In addition, the rumen eukaryotic metagenome will be prospected for potentially novel antimicrobials, which will then be tested *in vitro* for activity against bacterial pathogens. We currently have available, an eukaryotic metatranscriptome dataset at IBERS, which will be a great starting point for this work. A catalogue consisting of a reference set of rumen microbial genome sequences from cultivated rumen bacteria, methanogenic archaea, anaerobic fungi and ciliate protozoa is currently being developed through the Hungate1000 project (http://www.hungate1000.org.nz/). This provides an enormous amount of data for bioprospecting the rumen microbiome for novel antimicrobials, and will also be considered in the future.

CHAPTER SEVEN

Summary of thesis output

A variety of outputs have been created from the research carried out in this thesis. Thesis outputs have been presented at different international and national conferences in form of poster sessions, talks and submitted article manuscripts are outlined below beginning from the most recent.

7.1 Antimicrobial peptide patent application

We are currently in discussion with the Aberystwyth University Intellectual Property Office and will shortly be filling a patent application to the UK intellectual Property Office on the antimicrobial peptides identified in this study.

7.2 Articles

7.2.1 Oyama et al., (2015)

Oyama, LB., SE Girdwood, A. Cookson, N. Fernandez-Fuentes, JE Edwards, P Golyshin, O Golyshina, F Privé, R Mikut, K Hilpert, C Creevey and SA Huws (2015). **The rumen: an untapped goldmine for novel antimicrobial discovery**. *Manuscript submitted at Nature Biotechnology*.

Abstract:

Antimicrobial peptides (AMPs) are considered as future drug alternatives to combat the escalating problem of antimicrobial resistance. The community of competitive culturable and non-culturable bacteria in the rumen present an untapped goldmine for the discovery of antimicrobial compounds. We used metagenomic and bioinformatics techniques to prospect two rumen bacterial metagenomes for potentially novel AMPs. A total of 192 AMPs were identified. Seven of these (peptides 2, 3, 4, 5, 7, 8, and 15)

displayed potent antimicrobial activity (MICs between 32 to 64 µg/ml) against Epidemic methicillin resistant *Staphylococcus aureus* EMRSA-15 and *Escherichia coli* K12 and had no haemolytic activity against red blood cells. They also induced cytoplasmic leakage with some evidence of interference with the cell division mechanism. Our findings indicate that the rumen may provide viable alternative broad spectrum antimicrobial options for the prevention and treatment of multidrug resistant bacteria in the future.

7.2.2 Oyama et al., (2015b)

Oyama, LB., JA Crochet, SE Girdwood, A. Cookson, N. Fernandez-Fuentes, JE Edwards, P Golyshin, O Golyshina, F Privé, R Mikut, K Hilpert, C Creevey and SA Huws (2015). palG1: a novel protein from cow metagenome with activity against *Enterococcus faecalis*. *Manuscript in preparation.*

7.3 Offered oral presentations

7.3.1 Society for General Microbiology (SGM) Annual Conference, Birmingham, UK- March 30 to September 2. 2015.

A 15 minute oral presentation- title and abstract as below.

Broad spectrum antimicrobial peptides derived from a bovine rumen metagenome

Oyama. L. B., SE Girdwood, JE Edwards, P Golyshin, O Golyshina, F Privé, R Mikut, K Hilpert, C Creevey and SA Huws

Infections caused by multi drug resistant bacteria are exceptionally difficult to treat and overall the choice of currently available treatment options is very restricted. Urgent action is needed to increase the number of antimicrobials available for the treatment of these infections, a problem further compounded by the evolution of antimicrobial resistance. Antimicrobial peptides are a growing class of natural and synthetic peptides with a wide spectrum of targets including viruses, bacteria, fungi, and parasites gaining popularity as a potentially new option that can offer a solution to the problem.

A method of high throughput synthesis and screening was used to measure the antimicrobial activity of four novel antimicrobial peptides derived from a cow rumen metagenome library. These peptides show broad spectrum activity against and array of Gram positive and Gram negative organisms including *Pseudomonas aeruginosa*, EMRSA-15, *Enterococcus faecalis* and *Salmonella typhimurium* and were simultaneously be non-toxic to the host. Although, work on to investigate the killing kinetics and mode of action of these peptides is currently ongoing and still in the early stages, current results clearly demonstrate that these cow rumen derived peptides have potential for use in the treatment of bacterial infections, and the rumen is a potential a source of novel antimicrobial peptides that could provide templates for the design of novel therapeutics in the future.

7.3.2 First annual International Environmental 'Omics Synthesis conference, Cardiff, UK- September 9-11, 2013.

A 15 minute oral presentation: title and abstract below.

Use of functional and sequence based metagenomics to prospect the rumen metagenome for novel antimicrobials

The challenge of antimicrobial resistance in bacteria, coupled with the decrease in drug discovery rate is now a serious medical challenge. As such, there is a pivotal need for accelerated development of new antimicrobials. Metagenomic techniques allow the study of the whole microbiome (culturable and as yet unculturable bacteria), and allow the discovery of novel compounds. To date, turbomycin is the only antibiotic discovered using metagenomics but the potential to discover useful antibiotics from microbiomes in which bacteria commonly compete with each other is enormous. As such, we prospected a rumen fosmid based metagenome for antimicrobial activity against *Staphylococcus aureus, Escherichia coli, Listeria monocytogenes, Salmonella typhimurium,* and *Enterococcus faecalis*. Metagenomic clones (2µl) were pressed gently

using pin replicators onto Luria-Bertani agar plates which had 500µl of a pathogen (OD_{600}nm =1) spread evenly over the agar just prior. Plates were incubated overnight and the following day zones of clearing around the clones were noted as being indicative of clones which have gene inserts encoding antimicrobials. Antimicrobial activity was observed in 255 of 8448 clones, which are now undergoing high throughput pyrosequencing. Alongside functional metagenomics, we have mined existing rumen metagenomic datasets (Hess *et al* 2011; Brulc *et al* 2009) and pure culture genomic datasets using BLAST and BioEdit software, for homology to known antimicrobial gene sequences obtained from NCBI BLAST. Sequences with homology to antimicrobial genes across many antimicrobial classes, especially aminoglycosides, penicillins and carbapenems were found. We now aim to fully characterise antimicrobial genes identified using functional and sequence based metagenomics.

7.4 Posters

7.4.1 4th International Meeting on Antimicrobial peptides (IMAP), Graz Austria- September 29-30, 2014.

_pal_G1: A novel antimicrobial from cow rumen

The development of antibiotic molecules for use in the treatment of infections and for the upstream control of contamination has been declining over recent decades. Urgent action is needed to address this problem which is further compounded by the evolution of antibiotic resistance. Vancomycin-resistant *Enterococci* (VRE) have emerged as a major cause of nosocomial infections, and within this group *Enterococcus faecalis* causes the majority of human enterococcal infections.

In this study, a novel antimicrobial peptide ***pal*G1** discovered by functional and sequence based screening of a fosmid-based cow rumen metagenomic library was successfully expressed and tested for activity against *Enterococcus faecalis* JH2-2.

Growth of *E. faecalis* in Luria Bertani broth in the presence of ***pal*G1** was monitored over a 24 hour period. Bacterial growth was inhibited by ***pal*G1** (100 µg/ml) compared to medium with bacteria alone. Although, still in the early stages of characterisation, current results clearly demonstrate that ***pal*G1** has potential for use in the treatment of enterococcal infections, and the rumen is a potential a source of novel antimicrobial peptides that could provide templates for the design of novel therapeutics in the future.

palG1: A novel antimicrobial from cow rumen

LB Oyama[1], JA Crochet[1], JE Edwards[1], SE Girdwood[1], P Golyshin[2], O Golyshin[2], F Privé[1], C Creevey[1], SA Huws[1].

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Introduction

Urgent action is needed to address the declining rate of discovery and development of antibiotic molecules for use in the treatment of infections and the upstream control of contamination. This problem is further compounded by the increasing rate of antimicrobial resistance development.

Vancomycin-resistant *Enterococci* (VRE) have emerged as a major cause of nosocomial infections, and within this group *Enterococcus faecalis* causes the majority of human enterococcal infections[1].

In this study, a novel antimicrobial peptide ***palG1*** **(71 aa)** discovered by functional and sequence based screening of a fosmid-based cow rumen metagenomic clone library[2] was successfully expressed and tested for activity against *Enterococcus faecalis* JH2-2.

>***palG1***
MRLSHVCSQSSSKYPAGSCNLMTHDRAT
SSEHRSASRCDRVIRRFCRSDPASRYWRY
PVRGMRHGRCHTDW

Materials and Methods

Pilot agar based screen and 454 pyrosequencing
Following activity on preliminary agar based screen (results not shown), the clone carrying the gene *palG1* was sequenced using 454 FLX Pyrosequencing titanium technology.

Antimicrobial prediction and homology search
The open reading frames (ORFs) were characterised using ORF Finder on NCBI. Sequence analysis and homology searches were done using NCBI blastp and other bioinformatic software (See Table 1).

Cloning, gene expression and purification
The expression of ***palG1*** was undertaken using the pTrcHis TOPO® TA Expression kit.

Gel purified PCR amplified gene was cloned into the pTrcHis TOPO vector and expressed with a six-histidine tag at the N-terminus in *Escherichia coli* TOP10 *cells*.

The recombinant proteins were purified using affinity chromatography on nickel resin in native conditions in order to preserve their activity.

Antimicrobial activity studies
The antimicrobial activity of ***palG1*** was determined by a modified broth microdilution method[3]. The growth of *E. faecalis* in the presence of ***palG1*** was monitored over a 24 hour kinetic cycle (absorbance at OD_{600nm}) using a Hidex Sense microplate reader.

Results

See Table 1 for blast results and Figure 1 for amplified PCR product.

Confirmation of cloning reaction for the correct gene insert and orientation was done by tip-dip PCR and Sanger sequencing (see Figures 2 and 3).

The protein expression assay showed that expression was the most abundant 4 - 5h after induction of the clone cultures with 1 mM IPTG; as illustrated by SDS-PAGE results in Figure. 4(C).

The purification protocol routinely yielded 0.4 to 2 mg/mL of purified ***palG1*** protein from 40 mL lysate and protein was stored in 100mM HEPES (pH 7.5) (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) HEPES. Original elution buffer (400mM imidazole, 500mM NaCl, 20mM Tris.HCl pH 7.9) inhibited bacterial growth (results not shown).

See Figure 5. for SDS-gel of purification and Figure 6 for growth of *E. faecalis* in the presence of ***palG1***.

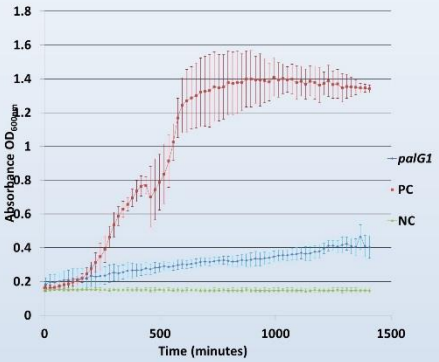

Figure 6. Antimicrobial activity testing of *palG1*. Growth of *E .faecalis* in the presence of ***palG1*** (100ug/ml) was markedly reduced and comparable to media only control (Luria Bertani LB broth). PC= Pathogen only, NC = no pathogen. **(From 5 replicates).**

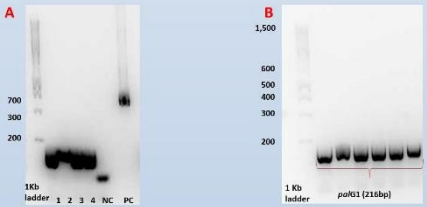

Figure 1. Amplified PCR product of *palG1*. A, visualization under UV For verification of PCR product (1-4 are replicatesof ***palG1***). NC = No DNA. PC= Control PCR DNA . **B**, Gel of PCR purified for cloning reaction (all six bands (replicates of **1** in **A**) were excised for cloning but only the best cloning reaction was continued.

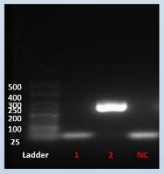

Figure 2. Cloning Confirmation of *palG1* by tip-dip PCR. PCR was done using the gene specific forward primer and the vector specific reverse primer. Expected size = 278bp (216bp of gene and 62bp of primer sequences either end. **2.** Clone with correct insert size.

Figure 3. Cloning Confirmation of *palG1* by Sanger sequencing. Alignment of Sanger sequence and expected sequence of *palG1* by Clustalw showed a perfect match indicating correct cloning of ***palG1***.

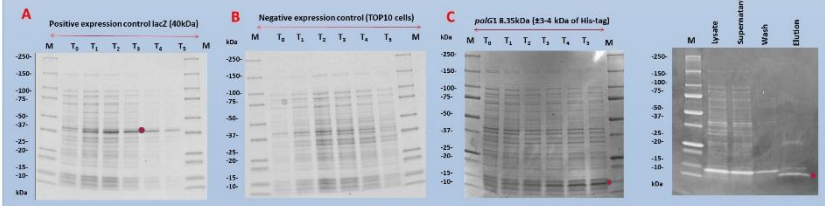

Figure 4. SDS-PAGE results of expression of *palG1* (C). A. positive control. **B** Negative control. Red dot shows band of protein of interest. **M,** Molecular weight marker. T_0, Time before induction. T_1-T_5, hourly time points after induction with Isopropyl β-D-1-thiogalactopyranoside (IPTG).

Figure 5. SDS-PAGE results for purification of *palG1*. Expected size is 8.35 (±3-4 kDa of His-tag).

Table 1. Antimicrobial prediction and homology search for *palG1*.

Most relevant NCBI blastp[4] hit to *palG1*				
Accession	**Function**	**Source**	**E-value**	**Identity**
WP_004942604.1	penicillin amidase	*Streptomyces mobaraensis*	7.6	37%

Significant Homologs to *palG1* on BACTIBASE[5]				
Identity	**E-value**	**Protein**	**Source**	**Activity**
30%	1.7	Enterocin 96	*Enterococcus faecalis*	Gram + Gram -
18%	4.8	Limocin M18	*Brevibacterium linens*	*Listeria* spp.

Conclusions

Although still in the early stages of characterisation, current results clearly demonstrate that ***palG1*** has potential for use in the treatment of enterococcal infections. This work also demonstrates the rumen is a potential source of novel antimicrobial peptides, which could provide templates for the design of novel therapeutics in the future.

Further characterization and testing of ***palG1*** is now in progress, in order to determine its potential for application in the treatment of enterococcal infections.

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4. Altschul S.F *et al.* (1997) Nucleic Acids Res. **25**:3389-3402.
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7.4.2 Rowett-INRA 2014, Gut Microbiology: from sequence to function, Aberdeen, UK- June 16-19, 2014.

From metagenomic sequences to antimicrobial function: mining the rumen microbiome.

Due to their broad-spectrum activity, antimicrobial peptides (AMPs) are considered as future drug alternatives to combat the problem of antimicrobial resistance in medicine. The community of competitive culturable and non-culturable bacteria in the rumen present a potential source of novel bioactive compounds. In this study, we screened a bovine fosmid based metagenomic library for antimicrobial activity against a range of pathogens including *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella typhimurium*, and *Enterococcus faecalis*. Metagenome clones were pressed onto a lawn of a pathogen on Luria-Bertani agar plates. After overnight incubation at appropriate temperatures, zones of clearing around the clones, indicative of antimicrobial gene inserts, were observed in 255 of 8448 clones. Twenty two putatively positive clones were sequenced using 454 FLX titanium technology. Sequence analysis and antimicrobial peptides/gene prediction were employed using bioinformatics techniques and AMP predicting software e.g. NCBI BLAST, ANTIBP2, AMPA, and CAMP. Numerous antimicrobial peptides across many antimicrobial peptide classes were identified in many of the open reading frames. Research into the antimicrobial activities of the predicted antimicrobial peptide sequences is currently ongoing. Preliminary antimicrobial activity studies of a few of these peptides show activity against human pathogens including *Salmonella typhimurium*. These novel AMPs will be fully characterized to determine their potential applicability in the treatment of pathogens.

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From metagenomic sequences to antimicrobial function: mining the rumen microbiome

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Introduction

Due to their broad-spectrum activity, antimicrobial peptides (AMPs) are considered as future drug alternatives to combat the problem of antimicrobial resistance in medicine[1]. The community of competitive culturable and non-culturable bacteria in the rumen present a potential source of novel bioactive compounds. Using modern metagenomic and bioinformatics techniques, it is possible to mine the entire microbial population in an environment[2]. Indeed the discovery of many bioactive compounds, and the antibiotic turbomycin from soil metagenome suggests that novel antimicrobial compounds can be identified using functional and sequence based metagenomics[2].

Aim: In this study, we screened a bovine fosmid based metagenomic library[3] (of 8,448 clones with an average insert size of 30 to 35kbp) for antimicrobial activity against a range of pathogens including *Staphylococcus aureus, Escherichia coli, Listeria monocytogenes, Salmonella typhimurium*, and *Enterococcus faecalis*.

MATERIALS AND METHODS

Pilot Agar Based Screen and 454 Pyrosequencing

Metagenomic clones (2µl) were gently pressed onto a lawn of 500µl of a pathogen (OD_{600}=1) on Luria-Bertani agar plates. Zones of clearing around the clones after overnight incubation at appropriate temperatures were indicative of antimicrobial gene inserts.
Twenty four putatively positive clones were sequenced using 454 FLX titanium technology.

Antimicrobial Prediction and homology search

The open reading frames (ORFs) were characterised using ORF Finder on NCBI. Sequence analysis, homology search and antimicrobial peptides/gene prediction were employed using bioinformatics techniques and AMP predicting software e.g using the AMPA antimicrobial Scanning system[4] the APD[5], CAMP[6], and the ANTIBP2 antibacterial peptide Prediction Server[7] as well as on NCBI blastp.

Antimicrobial Activity Studies

Antimicrobial studies of one antimicrobial peptide **SHALININ** and its predicted catalytic stretch **SHALININ 1 (**GL Biochem Shangai) is ongoing.
Minimum inhibitory concentration (MIC) was determined by a modified broth microdilution method[8] and growth kinetics was done in a 96 well plate reader.

RESULTS

- Many clones (255) showed agar based activity (See Table 1 and Figure 1).
- Numerous (a total of 197 large antimicrobial and small peptides) across many antimicrobial peptide classes were identified in many of the open reading frames (see Table 2 for a cross-section).
- Lag phase was prolonged in the presence of **SHALININ 1** compared to the growth control in *Salmonella typhimurium (See Figure 2).*

CONCLUSIONS

- Although antimicrobial testing of peptides is still ongoing, preliminary data suggests activity against an array of pathogenic bacteria.
- Activity in catalytic stretch means a reduced cost in peptide synthesis and affordability.
- The rumen provides a rich consortium of microorganisms, some of which have been reported to have antimicrobial properties. Preliminary data from this study show that this environment presents a potentially rich source for novel products, particularly antimicrobials.

Antimicrobial activity testing of all expressed/synthesised AMPs and full characterization to determine their potential applicability in the treatment of pathogens.

Table 1. **Agar based screening of rumen metagenomic clone library. ≥3 of 6 screens**

Pathogens	Number of clones with activity
E. coli (1)	64
S. aureus (2)	26
Sal. typhimurium (3)	139
Ent. faecalis (4)	7
L. monocytogenes (5)	19
1 x 2	1
1 x 3	29
1 x 2 x 3	1
2 x 3	15
2 x 5	1

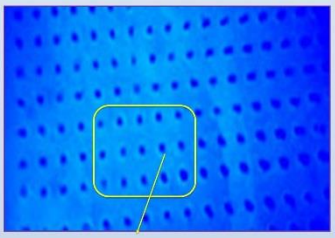

Figure 1. **Area on plate showing clones with activity (halos around them)**

Table 2. **Predicted Antimicrobial genes in fosmid samples by different AMP predicting databases: a cross section**

		AMPA		CAMP		ANTIBP2			APD2		
Peptide No	Peptide size (aa)	Propensity	% Probability in Non-AMP)	Class	AMP Probability	Class	Sub-family	Score	ID	Name	% Similarity
Pep1	35	0.2	1	AMP	0.997	Mammals	α-defensin	0.843	AP01669	Ipha-defensin	41.02
Pep27	15	0.207	2	AMP	0.563	Mammals	α-defensin	0.509	AP00191	Gomesin	42.1
Pep45	18	0.206	2	AMP	0.662	Mammals	α-defensin	0.406	AP01671	PhTD-3	47.61
Pep49	25	0.196	1	AMP	0.97	Mammals	α-defensin	0.225	AP01209	Retrocyclin-3	42.3
Pep106	26	0.203	1	AMP	0.93	Mammals	α-defensin	0.424	AP02178	Rattusin	40.62
Pep155	18	0.171	0	AMP	0.693	Mammals	α-defensin	0.951	AP00212	RatNP-1	40.9
Pep49	25	0.196	1	AMP	0.97	Mammals	α-defensin	0.225	AP01209	Retrocyclin-3	42.3
Pep70	21	0.203	1	AMP	0.566	Mammals	β-defensin	0.758	AP00518	Ib-AMP3	40
Pep115	16	0.166	1	AMP	0.84	Mammals	β-defensin	1.13	AP02348	Cerecidin A1	40.9
Pep62	18	0.186	0	AMP	0.742	Mammals	Histanin	0.166	AP01849	Andersonin-C1	40
Pep96	29	0.197	1	AMP	0.519	Mammals	Cathelicidin	0.22	AP01633	WLBU2	40
Pep130	20	0.21	2	AMP	0.64	Mammals	Cathelicidin	1.046	AP01239	Cathelicidin-BF	40
Pep139	22	0.188	0	AMP	0.866	Mammals	Cathelicidin	0.463	AP00706	Dahlein 5.6	44
Pep163	21	0.186	0	AMP	0.808	Mammals	Cathelicidin	0.67	AP00580	Nigrocin-2GRb	45.83
Pep147	16	0.211	3	AMP	0.939	Frog	Brevinin	0.36	AP01261	Riparin 2.1	41.17
Pep181	18	0.215	4	AMP	0.831	Frog	Brevinin	0.405	AP01578	Myxinidin	42.1
Pep11	16	0.228	11	AMP	0.994	Frog	Other	0.341	AP00156	BTD-2	47.61
Pep21	16	0.217	5	AMP	0.834	Frog	Other	0.769	AP00723	Decoralin	41.17
Pep28	17	0.156	0	AMP	0.999	Frog	Other	0.008	AP00519	Ib-AMP4	41.66

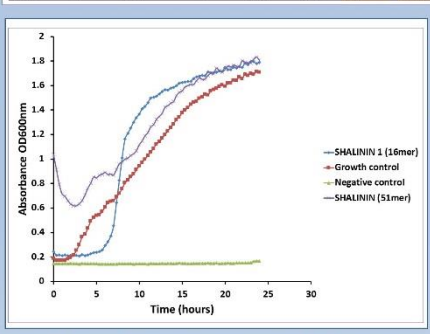

***Figure 2. Salmonella typhimurium*-24 hour Growth/ Killing Kinectics in LB Broth** (error bars too negligible to show).

Table 3. **MIC Values for *Sal. typhimurium***

Antimicrobial	Observed MIC (ug/ml)
SHALININ	≥2048
SHALININ 1	≥2048
Polymyxin B	2
Bac2A	256-1024
Vancomycin	512-1024
Tobramycin	16

References

1. Fedders H *et al.* (2010). International Journal of Antimcrobial Agents. **36**(3):264-266. **2**. Gillespie et al (2002). Applied and Environmental Microbiology **68**(9): 4301-4306. **3**. Privé F *et al.* (2013). PLoS ONE. **8**(8):e69076. **4**. Torrent M et al (2009). BMC Bioinformatics 11;10:373. **5**. Wang Z & Wang G (2004). Nucleic Acid Research 32, D590-D595. **6**. Thomas s et al (2010). Nucleic Acid Research 38: D774-D780. **7**. Lata S et al (2009). BMC Bioinformatics 11:S19. **8**. Wiegand I. et al. (2008). Nature Protocols. **3**(2): 163-175.

7.4.3 3rd International Meeting on Antimicrobial peptides (IMAP), London, UK- September 23-24, 2013.

Abundant antimicrobial peptides in cow rumen revealed by metagenomics.

Antimicrobial resistance is a serious challenge in medicine, which calls for an urgent need for the development and/or discovery of new antimicrobials. Antimicrobial peptides (AMPs) are currently being considered as future drug alternatives because of their broad-spectrum activity. The rumen consists of a huge community of competitive culturable and non-culturable organisms and so present a potential source for novel compound discovery. Using modern metagenomic and bioinformatics techniques, it is possible to mine the entire microbial population in an environment. Many bioactive compounds and the antibiotic, turbomycin have been discovered using these methods. Here, we screen a fosmid-based rumen metagenome library for antimicrobial genes. Sample sequences were obtained by 454 pyrosequencing and sequence analysis is currently ongoing. Antimicrobial peptides sequences were predicted using various bioinformatics and AMP predicting software including NCBI BLAST, ANTIBP2, AMPA, and CAMP. Numerous antimicrobial peptides across many antimicrobial peptide classes were identified in many of the sample open reading frames. These AMPs will be fully characterized to determine their novelty and potential applicability. Preliminary data from this study show that the rumen environment presents a potentially rich source for novel products, particularly antimicrobials peptides.

Abundant antimicrobial peptides in cow rumen revealed by metagenomics

Linda B. Oyama[1], Joan E. Edwards[1], Susan E Girdwood[1], Sharon A. Huws[1]

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Introduction

Antimicrobial resistance is a serious challenge in medicine, which calls for an urgent need for the development and/or discovery of new antimicrobials[1]. Antimicrobial peptides (AMPs) are key effector molecules of the innate immune system with broad-spectrum activity currently being considered as future drug alternatives[2]. Using modern metagenomic and bioinformatics techniques, it is possible to mine the entire microbial population in an environment[3]. Many bioactive compounds and the antibiotic, turbomycin have been discovered using these methods[4]. The rumen consists of a huge community of competitive culturable and non-culturable organisms and so present a potential source for novel compound discovery.

Aim: Prospect a rumen fosmid based metagenome library for antimicrobial activity against six pathogenic bacteria.

Materials and Methods

1. Agar Based Screen

Metagenomic clones (2µl) were gently pressed onto a lawn of 500µl of a pathogen (OD_{600}=1) on Luria-Bertani agar plates. Zones of clearing around the clones were indicative of antimicrobial gene inserts.

2. 454 Pyrosequencing/Sequence Analysis

24 randomly selected putatively positive fosmids were sequenced using the high throughput pyrosequencing Genome Sequencer FLX (454 Life Sciences) at Aberystwyth University. The pyrosequencing reads were assembled using the default parameters on the gsAssembler v2.5.3 software (Roche Life Sciences). The open reading frames (ORFs) were characterised using ORF Finder on NCBI. Antimicrobial peptide prediction and homology search were done using the AMPA antimicrobial Scanning system[5] the APD[6], CAMP[7], and the ANTIBP2 antibacterial peptide Prediction Server[8].

Results

1. Fosmid libraries consisted of 8,448 clones with an average insert size of 30 to 35kbp. Zones of clearing around the clones, indicative of antimicrobial gene inserts, were observed in 255 of 8448 clones after overnight incubation at appropriate temperatures. See Table 1.

2. Detailed analysis of fosmid open reading frames (ORFs) from pyrosequencing reads attributable to antimicrobial activity in putatively positive clones is ongoing. See Table 2 Preliminary results.

Table 1. Results from agar based screening of rumen metagenomic clone library. Showing only clones with growth and activity in ≥3 out of 6 screens

Pathogens	Number of clones with activity
Escherichia coli (1)	64
Staphylococcus aureus (2)	26
Salmonella typhimurium (3)	139
Enterococcus faecalis (4)	7
Listeria monocytogenes (5)	19
1 x 2	1
1 x 3	29
1 x 2 x 3	1
2 x 3	15
2 x 5	1

3. Difference in prediction methods and models between databases mean that a sequence may or may not be recognised as an AMP See. Table 3.

Table 2. Predicted Antimicrobial genes in fosmid samples (in all four AMP predicting databases)

Sample No	Clone ID/ORF	Protein size (aa)	ANTIBP2 Class/Sub-family/Score	CAMP probability	AMPA Propensity/ % probability in non-AMP	APD2 Chance/most similar homolog
22	SABPL29 H11/145	39	Mammals/ α-defensin / 0.325	AMP 0.868	0.199/1%	Yes/AP01669 PhD3 36.58%
23	SABPL29 I11/56	56	Mammals/ Cathelicidin/ 0.867	AMP 0.544	0.217/5%	Yes/AP00264 Chicken Heterophil Peptide 1 31.03%
	SABPL5 A1/180	54	Mammals/ Cathelicidin/ 0.417	AMP 0.505	0.193/0%	Yes/AP01592 BIN1b 33.33%
	SABPL5 A1/191	52	Mammals/ Cathelicidin/ 0.159	AMP 0.879	0.214/4%	Yes/AP00988 Arasin 2 30.9%
	SABPL5 A1/127	75	Plant/unknown/0.351	AMP 0.999	0.200/1%	Yes/AP02043 BjBD 31.81%
	SABPL5 A1/171	57	Mammals/ β-defensin/ 0.778	AMP 0.982	0.209/2%	Yes/AP02040 Alpha-purothionin alpha1-PTH 32.25%
	SABPL5 A1/214	48	Mammals/ β-defensin/ 1.148	AMP 0.634	0.195/1%	Yes/AP00043 Bovine Beta-defensin 8 34.54%
	SABPL5 A1/230	46	Mammals/ β-defensin/ 0.821	AMP 0.503	0.213/3%	Yes/AP00742 Chicken AvBD6 32.69%
2	SABPL5 A1/299	37	Mammals/ β-defensin/ 0.897	AMP 0.834	0.228/11%	Yes/ AP00709 Mytilus defensin (mytilin) A 34.04%
21	SABPL27 L17/109	35	Mammals/ β-defensin/ 0.047	AMP 0.659	0.206/2%	Yes/AP00442 defensin HANP-3 41.46%
	SABPL27 G15/61	77	Mammals/ β-defensin/ 1.527	AMP 0.722	0.220/6%	Yes/AP01157 Ixodidin 29.67%
16	SABPL27 G15/95	51	Mammals/ β-defensin/ 1.023	AMP 0.677	0.190/0%	Yes/AP00273 Buthinin 35.84%
13	SABPL12(2) G13/49	54	Mammals/ β-defensin/ 0.148	AMP 0.889	0.226/10%	Yes/AP01571 Strongylocin 1 32.78%
	SABPL27 H14/18	84	Mammals/ β-defensin/ 0.068	AMP 0.987	0.217/5%	Yes/AP00931 Uberolysin 29.67%
18	SABPL27 H14/45	46	Mammals/ β-defensin/ 0.777	AMP 0.685	0.206/2%	Yes/AP00745 Chicken LEAP-2 36.53%
	SABPL12(2) J19/67	73	Mammals/ β-defensin/ 1.458	AMP 0.968	0.163/0%	Yes/ AP01322 Ostrich AvBD7 32.43%
	SABPL12(2) J19/89	61	Mammals/ β-defensin/ 0.429	AMP 0.848	0.189/0%	Yes/ AP00650 Esculentin-1Plb 33.33%
	SABPL12(2) J19/97	55	Mammals/ β-defensin/ 0.444	AMP 0.778	0.205/2%	Yes/ AP00833 Human drosomycin-like defensin 31.66%
14	SABPL12(2) J19/153	39	Frog/Brevinin/0.538	AMP 0.580	0.232/14%	Yes/AP01507 Ranatuerin-2CPc 38.09%
	SABPL27 H14/34	59	Frog/Brevinin/0.208	AMP 0.499	0.202/ 1%	Yes/AP02110 Rugosin-LK2 31.25%
18	SABPL27 H14/21	72	Insect/Invertebrate	AMP 0.976	0.230/13%	Yes/AP01193 Halocin C8 31.76%
13	SABPL12(2) G13/69	45	Insect/Invertebrate	AMP 0.568	0.214/4%	Yes/AP01804/ Oxt 4a 32.6%
14	SABPL12(2) J19/46	91	Insect/Invertebrate	AMP 0.993	0.220/6%	Yes/AP00992 Snakin-2 30.61%
	SABPL12(2) J19/131	45	Insect/Invertebrate	AMP 0.568	0.214/4%	Yes/AP00981 PhD1 31.57%

Table 3. Predicted Antimicrobial genes in fosmid samples (showing differences in AMP prediction between databases)

Sample No	Clone ID/ORF	Protein size (aa)	CAMP probability	ANTIBP2 Class/Sub-family/Score	AMPA Propensity/ % probability in non-AMP
2	SABPL5 A1/107	89	AMP 0.536	Bacteria /unknown/0.146	
4	SABPL5 C17/54	40	AMP 0.751	Frog/Brevinin/1.145	
	SABPL27 H14/46	46	AMP 0.557	Frog/Brevinin/0.127	
18	SABPL27 H14/62	36	AMP 0.563	Bacteria/unknown/ 0.153	
14	SABPL 12 (2) J19/76	67	AMP 0.819	Frog/ Demaseptin/0.676	
19	SABPL27 L10/165	37		Mammals/α-defensin/ 1.270	0.171/0%
	SABPL5 A1/198	51		Mammals/β-defensin/0.820	0.162/0%
	SABPL5 A1/310	35		Mammals/β-defensin/ 0.646	0.157/0%
2	SABPL5 A1/174	57		Bacteria/unknown/0.153	0.233/15%
	SABPL29 I11/53	59		Bacteria/unknown/0.522	0.197/1%
	SABPL29 I11/77	48		Bacteria/unknown/0.346	0.229/12%
23	SABPL29 I11/97	38		Mammals/Cathelicidin/ 0.018	0.162/0%
2	SABPL5 A1/301	37		Mammals/Cathelicidin/0.198	0.211/3%
4	SABPL5 C17/64	33		Frog/Brevinin/0.037	
21	SABPL27 L17/102	37		Bacteria/unknown/0.098	
23	SABPL29 I11/101	38		Insect/Attacin/0.700	
	SABPL5 A1/244	43		Insect/cecropin/0.668	
	SABPL5 A1/308	35		Insect/Attacin/ 0.591	
	SABPL5 A1/222	46		Bacteria/unknown/ 0.442	
	SABPL5 A1/250	42		Bacteria/unknown/ 0.472	
	SABPL5 A1/307	36		Mammals/β-defensin/ 0.227	
	SABPL5 A1/321	33		Mammals/β-defensin/0.569	
	SABPL5 A1/325	33		Mammals/β-defensin/ 0.727	
	SABPL5 A1/172	57	AMP 0.947		0.16/0%
	SABPL5 A1/175	56	AMP 0.912		0.161/0%
	SABPL5 A1/261	40			0.170/0%
2	SABPL5 C17/59	39			0.169/0%
15	SABPL 19 A3/23	47			0.191/0%

Key

Bacteria	Mammals/β-defensin	Mammals/Cathelicidin	Mammals/α-defensin
Frog	Insect	Plant	Sample No with same colour = same sample

Future work

Antimicrobial peptides from positive clones will be expressed in a host and/or synthesized, fully characterised in order to determine their novelty and potential applicability.

Conclusion

The rumen provides a rich consortium of microorganisms, some of which have been reported to have antimicrobial properties. Preliminary data from this study show that this environment presents a potentially rich source for novel products, particularly antimicrobial peptides.

References

1. Coates, A. R. and Y. Hu (2007). British journal of pharmacology **152**(8): 1147-1154. **2** Fedders H et al (2010). International Journal of Antimicrobial Agents. **36**(3): 264-266. **3**. Handelsman, J. (2004). Microbiology and molecular biology reviews : MMBR **68**(4): 669-685. **4**. Gillespie et al (2002). Applied and Environmental Microbiology **68**(9): 4301-4306. **5**. Torrent M et al (2009). BMC Bioinformatics 11;10:373. **6**. Wang Z & Wang G (2004). Nucleic Acid Research 32, D590-D595. **7**. Thomas s et al (2010). Nucleic Acid Research 38: D774-D780. **8**. Lata S et al (2009). BMC Bioinformatics 11:519.

7.4.4 First annual International Environmental 'Omics Synthesis conference, Cardiff, UK- September 9-11, 2013.

Title and abstract are the same as specified in section 7.2.2

PRIFYSGOL ABERYSTWYTH UNIVERSITY

IBERS

Use of functional and sequence based metagenomics to prospect the rumen metagenome for novel antimicrobials

Linda B. Oyama[1], Joan Edwards[1], Martin Swain[1] and Sharon A. Huws[1]
[1]Institute of Biological Environmental and Rural Sciences
Aberystwyth University Wales United Kingdom SY23 3DA

Introduction

The challenge of antimicrobial resistance in bacteria, coupled with the decrease in drug discovery rate is now a serious medical challenge. Therefore, there is a pivotal need for accelerated development of new antimicrobials.[1]. Metagenomic techniques allow the study of the whole microbiome (culturable and as yet unculturable bacteria), and as such play a major role in novel compound discovery[2]. To date, turbomycin is the only antibiotic discovered using metagenomics[3] but the potential to discover useful antibiotics from microbiomes in which bacteria commonly compete with each other is enormous.

Aim: Prospect a rumen fosmid based metagenome library for antimicrobial activity against six pathogenic bacteria and mine existing rumen metagenomic datasets for homology to known antimicrobial gene sequences.

Materials and Methods

1. Agar Based Screen

Metagenomic clones (2µl) were gently pressed onto a lawn of 500µl of a pathogen (OD_{600}=1) on Luria-Bertani agar plates. Zones of clearing around the clones were indicative of antimicrobial gene inserts.

2. 454 Pyrosequencing/Sequence Analysis

24 randomly selected putatively positive fosmids were sequenced using the high throughput pyrosequencing Genome Sequencer FLX (454 Life Sciences) at Aberystwyth University.

The pyrosequencing reads were assembled using the default parameters on the gsAssembler v2.5.3 software (Roche Life Sciences). The open reading frames (ORFs) were characterised using ORF Finder on NCBI. Conserved domains in the amino acid sequences were analysed with Conserved-Domain search on NCBI and the Pfam database. Antimicrobial peptides and bacteriocin prediction and homology search were done using the APD[8], CAMP, BACTIBASE and BAGEL Databases, the ANTIBP2 antibacterial peptide Prediction Server[7], the AMPA antimicrobial Scanning system[6].

3. Mining existent Datasets

NCBI BLAST and BioEdit were used to mine existent rumen metagenomic and Pure culture genomic datasets for homology to known antimicrobial gene sequences obtained from NCBI.

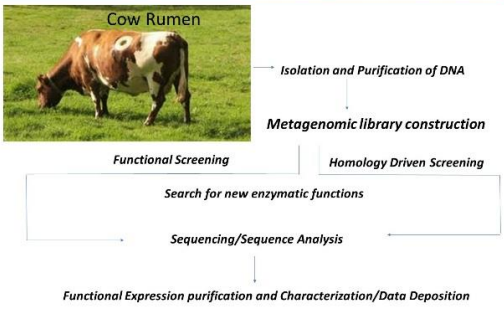

Figure 1. Metagenomic library construction and subsequent screening Procedures[4,9].

Results

1. Fosmid libraries consisted of 8,448 clones with an average insert size of 30 to 35kbp. Zones of clearing around the clones, indicative of antimicrobial gene inserts, were observed in 255 of 8448 clones after overnight incubation at appropriate temperatures. See Table 1.

Table 1. Results from agar based screening of rumen metagenomic clone library. Showing only clones with growth and activity in ≥3 out of 6 screens

Pathogens	Number of clones with activity
Escherichia coli (1)	64
Staphylococcus aureus (2)	26
Salmonella typhimurium (3)	139
Enterococcus faecalis (4)	7
Listeria monocytogenes (5)	19
1 x 2	1
1 x 3	29
1 x 2 x 3	1
2 x 3	15
2 x 5	1

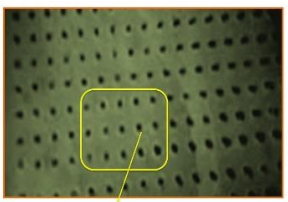

Figure 2. Area on plate showing clones with activity (halos around them).

2. Antimicrobial genes found in existent metagenomic[5] and genomic datasets. See Table 2 for sequences producing significant alignments.

Table 2. Antimicrobial genes found in existent metagenomic[5] and genomic datasets: sequences producing significant alignments

Antibiotic Class	Gene/Function /Source/Accession	Sequence length (aa)	% identity	E-value
Penicillins	pcbAB Penicillin Biosynthesis (*Nocardia lactamdurans*) CAA40561.1	3649	850/3062 (27%)	0
			825/2847 (29%)	0
	Acyl-coenzyme A/6-aminopenicillanic acid acyl-transferase (*Agromyces* sp. KY5R) BAE97628.1	376	59/211 (28%)	1.00E-10
Cephalosporins	CefD isopenicillin N epimerase (*N. lactamdurans*) AAA14022.1	398	72/191 (38%)	2.00E-31
Carbapenems	CarD (*Pectobacterium carotovorum*) AAD38232.1	376	109/324 (34%)	2.00E-51
	CarE (*P. carotovorum*) AAD38233.1	92	33/73 (45%)	4.00E-10
Aminoglycosides	gntA genatmicin biosynthesis (*Micromonospora echinospora*) CAF31430.1	420	124/313 (40%)	8.00E-60
			97/221 (44%)	1.00E-55
Aminoglycosides	genF gentamicin biosynthesis (*M. echinospora*) CAF31448.1	215	111/210 (53%)	5.00E-66
	KanB Kanamycin biosynthesis (*Streptomyces kanamyceticus*) CAE46938.1	427	103/220 (47%)	5.00E-60
Macrolides	EryBVI (*Saccharopolyspora erythraea*) involved in L-mycarose biosynthesis attached to the aglycones of several macrolide antibiotics. AAB84074.1	487	110/202 (54%)	1.00E-62

Table 3. Predicted Antimicrobial genes in fosmid samples

Clone/ORF	Protein size (aa)	NCBI BLASTN homology search			BACTIBASE	
		Putative function	Most similar homolog e-value	% Identity (overlapped aa)/CDD bit Score	Bacteriocin/e- value	% Identity
SABPL27 L13/6	571	peptidase C26 and renal dipeptidase family protein: hydrolytic metabolism of penem and carbapenem beta-lactam antibiotics	*Prevotella* sp. CAG:1185 CCY81602.1 e value 0.0	387/599(65%)	BAC193 Carocin D *Pectobacterium carotovorum* subsp. Carotovorum 0.96	35/150 (23%)
			Prevotella sp. CAG:520 CDB05864.1 e value 0.0	370/584(63%)		
SABPL5 A2/8	442	non-ribosomal synthesis of peptide antibiotics	PKS_TE[smart00824], Thioesterase peptide synthases 8.74e-03	36.05	BAC126 Pyocin S1 *Pseudomonas aureginosa* 0.74	10/24 (41%)
SABPL12(1) C3/50	80	colicin V production protein	*Pseudomonas putida* S16 NP_744149.1 e-value 1.4	19/61(31%	BAC172 Colicin-Ia *Escherichia coli* 0.52	6/22 (27%)
		bacteriocin production protein	*Pseudomonas putida* NBRC 14164 YP_008114872.1 e-value 2.0	19/61(31%)		
SABPL12(2) A3/63	65	Aminoglycoside N3-acetyltransferase	*Lactobacillus gasseri* ATCC 33323 YP_814852.1 e-value 3.7	19/49(39%)	BAC196 Lichenicidin A1 *Bacillus licheniformis* DSM 13 / ATCC 14580) 6.1	5/11 (45%)
			Halanaerobium saccharolyticum WP_005490435.1 e-value 4.2	20/55(36%		
SABPL27 L10/66	71	penicillin amidase	*Streptomyces mobaraensis* WP_004942604.1 e-value 5.0	16/43(37%)	BAC149 Enterocin 96 *Enterococcus faecalis* 1.7	3/10 (30%)
SABPL27 L10/73	68	beta-lactam antibiotic acylase	*Ornithinibacillus scapharcae* YP_004810705.1 e value 8.4	22/63(35%)	BAC164 Circularin A *Geobacillus kaustophilus* 3.2	9/17 (52%)

3. Detailed analysis of fosmid open reading frames (ORFs) from pyrosequencing reads attributable to antimicrobial activity in putatively positive clones is ongoing. See Tables 3 and 4 for Preliminary results.

Table 4. Predicted Antimicrobial peptides in fosmid samples

Clone/ORF	Protein size (aa)	ANTIBP2 Class Sub-family/Score	CAMP probability	AMPA Pipensity/% probability in non-AMP	APD2 Chance/most similar homolog
SABPL29 I11/56	56	Mammals Cathelicidin 0.867	AMP 0.544	0.217/5%	Yes/AP00264 Chicken Heterophil Peptide 1 31.03%
SABPL27 L17/145	39	Mammals α-defensin 0.325	AMP 0.868	0.199/1%	Yes/AP01669 PhD3 36.58%
SABPL27 G15/95	51	Mammals β-defensin 1.023	AMP 0.677	0.190/0%	Yes/AP00273 Buthinin 35.84%
SABPL12(2) J17/67	73	Mammals β-defensin 1.458	AMP 0.968	0.163/0%	Yes/ AP01322 Ostrich AvBD7 32.43%
SABPL12(2) J17/131	45	Insect Invertebrate defensin 0.574	AMP 0.568	0.214/4%	Yes/AP00981 PhD1 31.57%
SABPL12(2) J17/46	91	Insect Invertebrate defensin 0.784	AMP 0.993	0.220/6%	Yes/AP00992 Snakin-2 30.61%
SABPL12(2) J17/153	39	Frog Brevinin 0.538	AMP 0.580	0.232/14%	Yes/AP01507 Ranatuerin-2CPc 38.09%
SABPL27 H14/34	59	Frog/Brevinin/ 0.208	AMP 0.499	0.202/1%	Yes/AP02110 Rugosin-LK2 31.25%

Conclusion

The rumen provides a rich consortium of microorganisms, some of which have been reported to have antimicrobial properties. Preliminary data from this study show that this environment presents a potentially rich source for novel products, particularly antimicrobials.

Future work

Antimicrobial genes from positive clones will be expressed in a host and fully characterised in order to determine their novelty and potential applicability.

References

1. Coates, A. R. and Y. Hu (2007). British journal of pharmacology **152**(8): 1147-1154. 2. Handelsman, J. (2004). Microbiology and molecular biology reviews : MMBR **68**(4): 669-685. 3. Gillespie et al (2002). Applied and Environmental Microbiology **68**(9): 4301-4306. 4. Singh, B. K. (2010). Trends in biotechnology **28**(3): 111-116. 5. Hess et al (2011). Science **331**(6016): 463-467. 5. 6. Torrent M et al (2009). BMC Bioinformatics 11;10:373. 7. Lata S et al (2009). BMC Bioinformatics 11:S19. 8. Wang Z & Wang G (2004). Nucleic Acid Research 32, D590-D595. 9. Ferrer M et al (2009). J.Mol Microbiol Biotechnol 16:109-12. 10. Thomas s et al (2010). Nucleic Acid Research 38: D774-D780.

7.4.5 Society for General Microbiology (SGM) Autumn Conference, Sussex, UK- September 2-4, 2013.

Metagenomic technology as a tool for the discovery of novel antimicrobials

The increasing emergence of antimicrobial resistance in bacterial pathogens has led to a demand for novel antimicrobial compounds. Metagenomic techniques allow the study of the whole microbiome (culturable and as yet unculturable bacteria), and as such play a major role in novel compound discovery. Indeed, the discovery of turbomycin from soil metagenome suggests that novel antimicrobial compounds can be identified using metagenomics. We prospected a rumen fosmid based metagenome library for antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella typhimurium*, and *Enterococcus faecalis*. Metagenomic clones (2µl) were gently pressed onto a lawn of 500µl of a pathogen (OD_{600}nm =1) on Luria-Bertani agar plates. Zones of clearing around the clones, indicative of antimicrobial gene inserts, were observed in 255 of 8448 clones after overnight incubation at appropriate temperatures. These putatively positive inserts are now in the process of being pyrosequenced. Rumen metagenomic and pure culture genomic datasets have also been mined, using BLAST and BioEdit, for homology to known antimicrobial gene sequences obtained from NCBI BLAST. Numerous hits were identified across many antimicrobial classes, especially aminoglycosides, penicillins and carbapenems. These antimicrobial genes will be expressed in a host and fully characterised in order to determine their novelty and potential applicability.

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Metagenomic technology as a tool for the discovery of novel antimicrobials

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Introduction

The increasing emergence of antimicrobial resistance in bacterial pathogens has led to a demand for novel antimicrobial compounds[1]. Metagenomic techniques allow the study of the whole microbiome (culturable and as yet unculturable bacteria), and as such play a major role in novel compound discovery[2]. Indeed, the discovery of turbomycin from soil metagenome suggests that novel antimicrobial compounds can be identified using metagenomics[3]. In this study, we prospected a rumen fosmid based metagenome library for antimicrobial activity against *Staphylococcus aureus, Escherichia coli, Listeria monocytogenes, Salmonella typhimurium,* and *Enterococcus faecalis*.

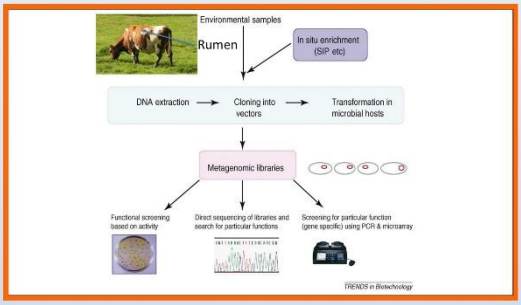

Figure 1. Metagenomic library construction and subsequent screening Procedures[4].

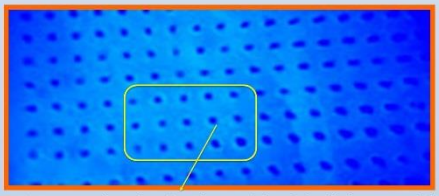

Figure 2. Area on plate showing clones with activity (halos around them)

Materials and Methods

1. Agar Based Screen

Metagenomic clones (2µl) were gently pressed onto a lawn of 500µl of a pathogen (OD_{600}=1) on Luria-Bertani agar plates. Zones of clearing around the clones were indicative of antimicrobial gene inserts.

2. 454 Pyrosequencing

Putatively positive fosmids were sequences using the high throughput pyrosequencing Genome Sequencer FLX (454 Life Sciences) at Aberystwyth University. and sequences were obtained according to the manufacture's protocol.

3. NCBI BLAST and BioEdit were used to mine existent rumen metagenomic and Pure culture genomic datasets for homology to known antimicrobial gene sequences obtained from NCBI.

Table 1. Results from agar based screening of rumen metagenomic clone library. Showing only clones with growth and activity in ≥3 out of 6 screens

Pathogens	Number of clones with activity
E. coli (1)	64
S. aureus (2)	26
Sal. typhimurium (3)	139
Ent. faecalis (4)	7
L. monocytogenes (5)	19
1 x 2	1
1 x 3	29
1 x 2 x 3	1
2 x 3	15
2 x 5	1

Table 2. Antimicrobial genes found in existent metagenomic[5] and genomic datasets: sequences producing significant alignments

Antibiotic Class	Gene/Function /Source/Accession	Sequence length (aa)	% identity	E-value
Penicillins	pcbAB Penicillin Biosynthesis (*Nocardia lactamdurans*) CAA40561.1	3649	850/3062 (27%)	0
			825/2847 (29%)	0
	Acyl-coenzyme A/6-aminopenicillanic acid acyl-transferase (Agromyces sp. KY5R) BAE97628.1	376	59/211 (28%)	1.00E-10
Cephalosporins	CefD isopenicillin N epimerase (*N. lactamdurans*) AAA14022.1	398	72/191 (38%)	2.00E-31
Carbapenems	CarD (*Pectobacterium carotovorum*) AAD38232.1	376	109/324 (34%)	2.00E-51
	CarE (*P. carotovorum*) AAD38233.1	92	33/73 (45%)	4.00E-10
Aminoglycosides	gntA genatmicin biosynthesis (*Micromonospora echinospora*) CAF31430.1	420	124/313 (40%)	8.00E-60
			97/221 (44%)	1.00E-55
Aminoglycosides	genF gentamicin biosynthesis (*M. echinospora*) CAF31448.1	215	111/210 (53%)	5.00E-66
	KanB Kanamycin biosynthesis (*Streptomyces kanamyceticus*) CAE46938.1	427	103/220 (47%)	5.00E-60
Macrolides	EryBVI (*Saccharopolyspora erythraea*) involved in L-mycarose biosynthesis attached to the aglycones of several macrolide antibiotics. AAB84074.1	487	110/202 (54%)	1.00E-62
			207/449 (46%)	1.00E-112

Results

- Fosmid libraries consisted of 8,448 clones with an average insert size of 30 to 35kbp. Zones of clearing around the clones, indicative of antimicrobial gene inserts, were observed in 255 of 8448 clones after overnight incubation at appropriate temperatures (See Table 1).
- Detailed analysis of fosmid open reading frames (ORFs) from pyrosequencing reads attributable to antimicrobial activity in putatively positive clones is ongoing.

Conclusion

The rumen provides a rich consortium of microorganisms, some of which have been reported to have antimicrobial properties. Preliminary data from this study show that this environment presents a potentially rich source for novel products, particularly antimicrobials.

Future work

Antimicrobial genes from positive clones will be expressed in a host and fully characterised in order to determine their novelty and potential applicability.

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