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**Eluding antibiotic resistance: capitalizing on antimicrobial peptides interaction with
the lipid bilayer**

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A

DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
M.D. Anderson Cancer Center
Graduate School of Biomedical Sciences
In Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

Danielle M. McGrath, B.S.
Houston, TX

August, 2011

DEDICATION

To my Mother who fueled my passion for learning with a never ending supply of Nancy Drew and to my Father who always knew I would do it.

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The scientific education and training that I received during my years of pre-graduate schooling established the foundation and desire necessary to complete this journey. Maribeth Watwood my undergraduate advisor, along with certain laboratory members namely Bre Espen and Scott Clingenpeel were instrumental in my decision to pursue a graduate education.

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**Eluding antibiotic resistance: capitalizing on antimicrobial peptides interaction with
the lipid bilayer**

Publication No. _____

Danielle M. McGrath

Supervisory Professor: Renata Pasqualini, Ph.D.

It is widely accepted that the emergence of drug-resistant pathogens is the result of the overuse and misuse of antibiotics. Infectious Disease Society of America, Center for Disease Control and World Health Organization continue to view, with concern, the lack of antibiotics in development, especially those against Gram-negative bacteria.

Antimicrobial peptides (AMPs) have been proposed as an alternative to antibiotics due to their selective activity against microbes and minor ability to induce resistance. For example, the Food and Drug Administration approved Daptomycin (DAP) in 2003 for treatment of severe skin infections caused by susceptible Gram-positive organisms. Currently, there are 12 to 15 examples of modified natural and synthetic AMPs in clinical development. But most of these agents are against Gram-positive bacteria. Therefore, there is unmet medical need for antimicrobials used to treat infections caused by Gram-negative bacteria.

In this study, we show that a pro-apoptotic peptide predominantly used in cancer therapy, (KLAKLAK)₂, is an effective antimicrobial against Gram-negative laboratory strains and clinical isolates. Despite the therapeutic promise, AMPs development is hindered by their susceptibility to proteolysis. Here, we demonstrate that an all-D enantiomer of (KLAKLAK)₂, resistant to proteolysis, retains its activity against Gram-negative pathogens. In addition, we have elucidated the specific site and mechanism of action of _D(KLAKLAK)₂ through a repertoire of whole-cell and membrane-model assays.

Although it is considered that development of resistance does not represent an obstacle for AMPs clinical development, strains with decreased susceptibility to these compounds have been reported. Staphylococci resistance to DAP was observed soon after its approval for use and has been linked to alterations of the cell wall (CW) and cellular membrane (CM) properties. Immediately following staphylococcal resistance, Enterococci resistance to DAP was seen, yet the mechanism of resistance in enterococci remains unknown. Our findings demonstrate that, similar to *S. aureus*, development of DAP-resistance in a vancomycin-resistant *E. faecalis* isolate is associated with alterations of the CW and properties of the CM. However, the genes linked to these changes in enterococci appear to be different from those described in *S. aureus*.

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List of abbreviations

AADs	aminoglycoside acetyltransferases
AMEs	aminoglycoside modifying enzymes
AMP	antimicrobial peptide
APHs	aminoglycoside phosphotransferases
AS	aggregation substance
BHI	brain-heart infusion broth
CA-MHB	cation-adjusted Mueller Hinton broth
cAMP	cyclic adenosine monophosphate
CF	cystic fibrosis
CFU	colony forming units
cGMP	cyclic guanosine monophosphate
CL	cardiolipin
CM	cell membrane
CTFR	trans-membrane conductance regulator
CV	crystal violet
CW	cell-wall
DAEC	diffusely adherent <i>E. coli</i>
DAP	daptomycin
diSC3(5)	3,3'-dipropylthiadicarbocyanine iodide
EAEC	enteroaggregative <i>E. coli</i>
EDTA	ethylenediaminetetraacetic acid

EHEC	enterohaemorrhagic <i>E. coli</i>
EIEC	enteroinvasive <i>E. coli</i>
EPEC	enteropathogenic <i>E. coli</i>
ESBL	extended spectrum β -lactamase
ETEC	enterotoxigenic <i>E. coli</i>
FDA	Food and Drug Administration
FRET	fluorescence resonance energy transfer
HA-MRSA	hospital-associated methicillin resistant <i>S. aureus</i>
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGT	horizontal gene transfer
HUS	hemolytic uremic syndrome
KCL	potassium chloride
L-PG	lysyl-phosphatidylglycerol
LB	Luria Bertani
LT	heat-labile enterotoxin
MALDI	matrix-assisted laser desorption/ionization time-of-flight
MAP	mitogen-activated protein
MBL	metallo- β -lactamases
MDR	multi-drug-resistant
MIC	minimum inhibitory concentration
MMR	mismatch repair
MNEC	meningitis/sepsis-associated <i>E. coli</i>
MOPS	3-(N-morpholino) propanesulfonic acid

MS	mass spectrometry
MSCRAMMs	microbial surface components recognizing adhesive matrix molecules
NBD-PE	N-(7-nitro-2,1,3-benzoxaidasol-4-yl)-PE
NF-kB	nuclear factor kappa B
OXA	oxacillinases
PBPs	penicillin binding proteins
PBS	phosphate buffer solution
PC	1,2-distearoyl-sn-glycero-3-phospho-L-serine
PE	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
PG	1,2-dioleoyl-sn-glycero-3-phosphatidylglycerol
PKC	protein kinase C
PS	1,2-dioleoyl-sn-glycero-3-phospho-L-serine
QS	quorum sensing
Rh-PE	N-(rhodamine B sulfonyl)-PE
STs	heat-stable enterotoxin
TEM	transmission electron microscopy
TFA	trifluoroacetic acid
TOF	time-of-flight
TSS	toxic shock syndrome
TTSS	type III secretion system
UPEC	uropathogenic E. coli
VRE	vancomycin-resistant enterococci

VT

verocytotoxin

Chapter I

Introduction

Chapter I

Introduction

Antimicrobial Resistance

Resistance to single antibiotics is not a new phenomenon. Although, the first antibiotic -- penicillin-- was discovered in 1928, large-scale production did not start until 1940. By 1944, staphylococci had become resistant to penicillin (1). Partially, this phenotype was due to the secretion of a specific enzyme, penicillinase, able to degrade penicillin (2, 3). Ever since, the introduction of new classes of antibiotics increased the selective pressure on specific microbial populations resulting in the emergence of multi-drug-resistant (MDR) organisms. These microbes are resistant to many classes of antibiotics due to several different types of resistance mechanisms including: enzymatic mechanisms of drug modification, enhanced efflux pump expression, altered membrane permeability, and mutated drug targets.

Genetics of antibiotic resistance

Bacteria can become resistant to antibiotics through intrinsic or acquired mechanisms. The mechanisms of acquired resistance involve chromosomal mutations, horizontal gene transfer of resistance determinants (4) or a combination of these two means.

Spontaneous mutations

Spontaneous mutations occur randomly in the form of incorrect repair of DNA damage or replication errors in actively dividing cells (5). Point mutations confer resistance when they are able to produce a resistance phenotype, and allow growth of the bacteria despite the presence of antibiotics (2, 6). Mutations leading to resistance usually map to the target of antibiotics. For example, mutations in the genes encoding RpoB and topoisomerases, which are the targets of rifampicins and fluorquinolones, confer resistance to these molecules (7, 8). In addition, mutations in the promoter regions of regulatory genes (9, 10) encoding for efflux or uptake systems also lead to resistance. In the case of *P. aeruginosa*, reduced expression of the OprD porin leads to resistance to carbapenems (11). Mutations that lead to the up-regulation of antibiotic-inactivating-enzymes also increase antimicrobial resistance.

Hypermutator phenotype

Normally, spontaneous mutation rates due to errors produced in the DNA replication process in a species remain low due to the activity of molecular mechanisms that act to repair and protect DNA. However, bacteria with high mutation rates have been identified. The majority of the bacteria with high mutation rates (hypermutators) have an inactive *mutS* or *mutL* gene, which leads to a defective mismatch repair (MMR) system (12). Usually, MMR eliminates biosynthetic errors and prevents recombination by non-identical DNA sequences (13). Hypermutable phenotypes act by accelerating the emergence mutations responsible for antibiotic resistance (7, 14, 15).

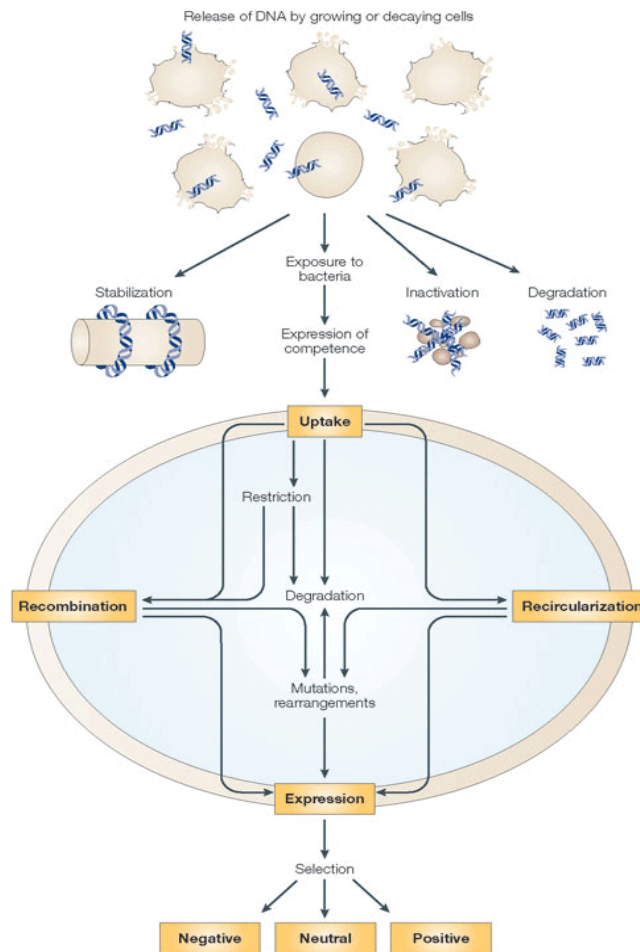
Adaptive mutagenesis

In addition to mutations that occur due to errors during the DNA replication process, mutations can also occur in non- or slowly dividing cells. These mutations, called adaptive mutations, differ from spontaneous mutations based on their occurrence only in the presence of non-lethal selective pressure that favors them (4), such as a sub-inhibitory drug concentration which often arises when treatment doses of antibiotics are not followed.

Horizontal gene transfer (HGT)

The first evidence of HGT was the recognition of the transfer of virulence determinants between pneumococci in infected mice (16). HGT can be the result of one of three mechanisms: (i) natural transformation (Figure 1-1) defined by stable uptake, integration and expression of extracellular DNA; (ii) transduction mediated by bacteriophages or (iii) conjugative transfer (Figure 1-2), where a cell-to-cell junction or pillus mediates transfer.

Natural transformation occurs when competent bacteria uptake free extracellular DNA molecules present in the environment. Extracellular DNA is released from disrupted or decomposing cells, bacteriophage, or naturally excreted from living cells (17). Competence is most often a time-limited physiological state involving up to 50 proteins (17). It is usually regulated in response to specific environmental conditions, although some, most importantly pathogenic bacteria, are naturally competent. The conditions that lead to bacterial cell competence are often altered growth conditions, cell

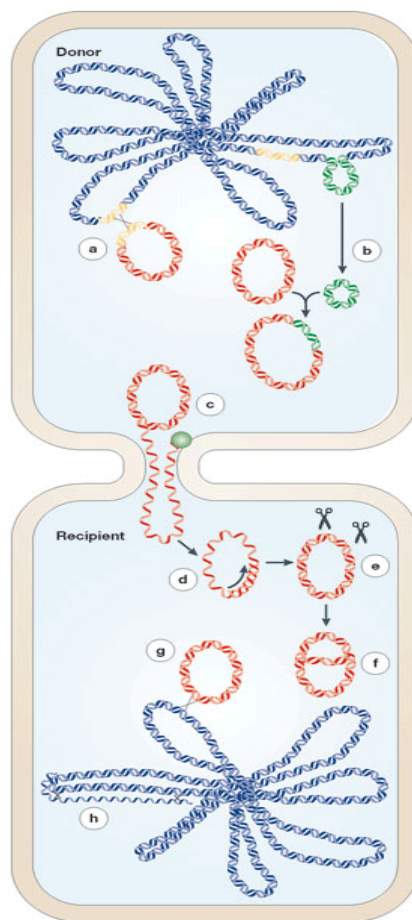


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Figure 1-1. Natural Transformation

“The steps involved in this process include the release of extracellular DNA into the environment and the uptake of DNA into the cytoplasm of the recipient bacterial cell that has developed a regulated physiological state of competence. Following uptake, for the transferred DNA to persist it must integrate into the bacterial genome through homologous recombination or by sequence-independent, illegitimate recombination. Plasmids that succeed in reconstituting a replication-proficient form do not need to integrate into the host genome.”

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Figure 1-2. Conjugative transfer.

“In the donor, the events depicted are: a, integration of the plasmid into the chromosome by recombination between insertion sequence elements; b, movement of a transposable element through a circular intermediate from the chromosome to the plasmids; c, initiation of rolling-circle replication at the mating-pair apparatus. In the recipient cell, the events depicted are: d, recircularization; e, attack by restriction endonucleases (scissors); f, replication; g, integration into the chromosome by an illegitimate Campbell recombination; h, recombination between transferred chromosomal DNA and the resident chromosome.”

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density, starvation or nutrient access. Competence in the bacterial population can range anywhere from 0 to 100%. Transformation begins with non-covalent binding of DNA to the cell surface, followed by translocation across the inner membrane. With the exception of plasmids, DNA must integrate into the bacterial genome to persist.

The transfer of DNA from donor bacteria to recipient bacteria is mediated by infection with a bacteriophage is called transduction. There are 2 types of transduction: generalized and specialized. Generalized transduction occurs when an individual lytic phage carrying different sets of genes pertaining to the bacterial genome transfers the foreign DNA to another bacterium (18). Specialized transduction occurs when individual phage transfer the same genetic information, usually present adjacent to the insertion site. A lysogenic phage inadvertently becomes lytic and improperly expels itself from the genome carrying the flanking host DNA sequences. Upon infection, the extraneous DNA is transferred to another bacterium. (18).

Conjugation occurs when all or part of the donor plasmid is transferred through direct contact between donor and recipient bacteria (Figure 1-1). The ability of the donor bacteria to transfer its genetic material is determined by specific conjugative plasmids; where strains of bacteria with the plasmid function as donors and those that lack the plasmid function as recipients (18).

Modes of antibiotic resistance

Antibiotic resistance emerges by one of 4 mechanisms: (i) direct inactivation of the antibiotic molecule (19), (ii) alteration of the target of the antibiotic (20), (iii) reducing the concentration of the drug without modifying the drug itself, through efflux

pumps and outer membrane permeability (21), iv) bypassing the inactivation of a given enzyme or cellular process (22).

Antibiotic inactivation

Antibiotics can be inactivated by the production of enzymes that degrade or modify the antibiotic molecule through hydrolysis, group transfer or redox mechanisms (4). A classical example of inactivation by hydrolysis are the chromosomally and plasmid-encoded β -lactamase enzymes that cleave the β -lactam ring of penicillin and cephalosporin antibiotics. In addition to the over 200 β -lactamases identified to date (4), over 180 extended-spectrum β -lactamases have been discovered that confer resistance to all penicillins, third generation cephalosporins and aztreonam (4). Esterases and epoxidases confer resistance to macrolides and fosomycin, respectively (23, 24, 25). Transferases inactivate antibiotics by chemical substitution, leading to altered binding to the antibiotic target. Oxidation and reduction of antibiotics has been infrequently identified in pathogenic bacteria (26).

Target modification

Modification of the target comes in the form of peptidoglycan structure alteration, protein synthesis interference and DNA synthesis interference, which leads to the inability of the antibiotic to bind. Peptidoglycan is an essential component of the bacterial cell-wall (CW), and therefore the enzymes involved in its synthesis and assembly serve as excellent targets for antibiotics. Often, penicillin-binding proteins are mutated; these mutations lead to decreased affinity of β -lactam antibiotics (27, 28, 29).

In addition to penicillin binding proteins, often bacteria will acquire mutations causing alterations of the C-terminal site of peptidoglycan pre-cursors, which are common targets of glycopeptides.

Mutations in the bacterial 50S ribosomal subunit are a common form of resistance to many antibiotics whose mechanism of action is interfering with protein metabolism. For example, a post-transcriptional modification to the 23S rRNA in the 50S ribosomal subunit confers resistance to macrolides, lincosamides and streptogramin B (30). In addition to the 23S subunit, mutations in the 16S rRNA gene are also common and confer resistance to aminoglycosides.

Topoisomerase IV and DNA gyrase contain sites of interaction for fluoroquinolones. This interaction causes a cessation of DNA replication and transcription. Fluoroquinolone resistance is often the result of mutations in the structural genes encoding for these molecules, leading to the inability of antibiotics to bind (31).

Outer membrane permeability and efflux pumps

Decreased outer membrane permeability results in reduced antibiotic uptake. When coupled with efflux pumps increased activity, the concentration of antibiotic is kept at very low levels inside the cell. Efflux pumps affect all classes of antibiotics and vary widely in their specificity and mechanism (32, 33). Some pumps are very drug specific, while others are multi-drug transporters and efflux a wide variety of unrelated antibiotics (34).

Antibiotics must cross the outer membrane of Gram-negative bacteria to reach their cellular target. This process is accomplished by either diffusion or self-promoted

uptake (4). Diffusion takes place through porins or directly through the bilayer.

Mutations in porins such as number, size and selectivity will greatly alter the rate of diffusion of antibiotics (35, 36, 37, 38, 39).

Pseudomonas aeruginosa

Microbiology

Pseudomonas aeruginosa is a non-fermentive, motile Gram-negative rod that is ubiquitous in soil and water (40, 41). In nature, *P. aeruginosa* is found in many forms such as a biofilm, attached to a surface or substrate, or in a planktonic form, as a unicellular organism. It is tolerant to a wide variety of physical conditions, including temperature, and is resistant to high concentrations of salts, dyes, weak antiseptics, and many commonly used antibiotics.

Disease

P. aeruginosa is recognized as a major cause of nosocomial bacteremia and infections associated with invasive devices, mechanical ventilation, burn wounds, or surgeries (42). The worldwide emergence of MDR nosocomial clones has added significantly to the ominous prognosis of *P. aeruginosa* infections.

Pathogenesis

P. aeruginosa has a wide host of virulence factors and produces many toxins and surface components associated with virulence (43, 44, 45). *P. aeruginosa* undertake two

main mechanisms of pathogenic behavior: i) remains confined as a chronic infection, for example, in the lungs of cystic fibrosis (CF) patients or ii) invades tissues causing bacteremia and pneumonia, usually resulting in septic shock and death.

In the CF chronic lung infection, *P. aeruginosa* usually down-regulates its toxins (46), loses its 'O' side chains (47), loses its flagellum and pilli (48, 49) and expresses alginate (mucoid coat) on the surface to protect itself from phagocytosis (50). Although the flagellum of *P. aeruginosa* are down regulated in chronic infections, it plays an important role in dispersal and adhesion to host cells (51, 52, 53, 54). These mutations are important to the persistence of *P. aeruginosa* because production of these factors would elicit an immune response, and without them the organism is able to evade the immune mechanisms in the lungs.

P. aeruginosa employs a secretion system composed of 43 genes called the type III secretion system (TTSS), to inject specific toxins directly into the host cell (55), (56). Four effector proteins have been identified: ExoS, ExoT, ExoU and ExoY of which ExoS, ExoT and ExoU have been related to virulence (57). ExoS and ExoT are found in 70 to 100% of clinical isolates, respectively (58) and contribute to phagocytosis inhibition. Their mechanism of action involves disruption of host cell actin cytoskeleton resulting in cell death (59). The ExoU gene is found in only 30% of clinical isolates (59) and encodes for phospholipase A₂ (PLA₂) (60, 61). The PLA₂ activity of ExoU leads to dissolution of the plasma cell membrane, followed by rapid cell death (60, 61, 62) and a large increase in production of prostaglandins (63) leading to increased tissue damage and excessive inflammation.

Quorum sensing (QS) is another important virulence mechanism of *P.*

aeruginosa. When bacteria reach high density, they secrete a QS effector molecule called autoinducer, which is sensed by neighboring bacteria (64). In *P. aeruginosa*, the QS system regulates many processes from production of toxins to biofilm formation (65, 66, 67, 68, 69, 70, 71).

Mechanisms of Antibiotic Resistance

P. aeruginosa is one of the leading causes of nosocomial infections worldwide (72). Besides intrinsic resistance determinants, *P. aeruginosa* has the ability to acquire further resistance mechanisms making infection with this pathogen often hard to treat. Almost all known mechanisms of antimicrobial resistance have been demonstrated in *P. aeruginosa*. Often these resistance mechanisms are not present singularly but in concert with each other, thereby conferring a multi-resistant phenotype.

The most prevalent mechanism of resistance to β -lactams is enzyme production. In 1980, Ambler (73) described the molecular classification of β -lactamases based on the nucleotide and amino acid sequences of the enzymes. There are 4 recognized classes named A-D, correlating with their functional classification (74). Classes A, C and D act through a serine based mechanism, class B are known as the metallo- β -lactamases (MBLs) and require zinc for their action. *P. aeruginosa* expresses significant numbers of enzymes pertaining to all classes (A-D).

AmpC β -lactamase is an inducible chromosomally encoded molecular class C enzyme that is usually produced in low quantities by *P. aeruginosa* (75). However, in the presence of inducing β -lactams its production increases 100-1000 times (76). Four

carbenicillin hydrolyzing β -lactamases belong to molecular class A (77). To date, *P. aeruginosa* has not been found to have plasmid mediated cephalosporinases.

PER-1 was the first fully characterized extended spectrum β -lactamase (ESBL). It was found to be chromosomally encoded but has since been reported to be plasmid mediated (78).

In addition to class A ESBLs, many class D β -lactamases, also known as oxacillinases (OXA), have been identified in *P. aeruginosa*. The classical OXA enzyme can confer resistance to carboxypenicillins and ureidopenicillins but not to ceftazidime (77). However, the OXA types identified in *P. aeruginosa*, are able to hydrolyze ceftaxidime, and therefore have great clinical importance. Moreover, this type of OXA is also able to hydrolyze many other antibiotics. Their spectrum includes: cefotaxime, cefepime, cefepime aztreonam, and moxalactam. In addition to a wide spectrum of effectiveness, these OXA types are at most instances not suppressed by β -lactamase inhibitors (79).

P. aeruginosa can also produce carbapenem-hydrolyzing enzymes that belong to the molecular class B (74), known as carbapenemase or MBLs (78). Production of carbapenemases confers resistance to all β -lactams, except the monobactam and aztreonam.

The high molecular mass proteins OprM, OprJ, OprN present in the outer membrane of *P. aeruginosa* contribute to low membrane permeability (80) and act as components of active efflux systems with wide substrate specificity (81). Efflux also contributes to the development of a multi-resistant phenotype and is mediated by four genetically different three-component efflux systems: (81, 82) MexA–MexB–OprM,

MexC–MexD–OprJ, MexE–MexF–OprN, and MexX–MexY–OprM. These three component systems have similar structures with the first component operating as an energy dependant pump located in the cytoplasmic membrane (MexB, MexD, MexF and MexY). The second component functions as an outer membrane protein (OprM, OprJ, OprN and OprM). The third protein functions as a linker between the other two proteins and is located in the periplasmic space (MexA, MexC, MexE and MexX) (82).

OprD is a protein that forms specific channels that allow the entry of basic amino acids and carbapenems into the cell (81). A deficiency of the OprD porins is observed in imipenem-resistant *P. aeruginosa* isolates (83). Rarely, it has been observed that *P. aeruginosa* can alter its penicillin binding proteins, the active site for β -lactam antibiotics (83).

Resistance to aminoglycosides comes in the form of enzyme modification, and to a lesser extent, decreased outer membrane permeability, target modification and active efflux (84, 85, 86). There are 3 classes of aminoglycoside modifying enzymes (AMEs); aminoglycoside phosphotransferase (APHs), aminoglycoside adenylyltransferases (AADs) and aminoglycoside acetyltransferases (AACs). AMEs function by decreasing the binding affinity of the aminoglycoside to the bacterial 30S ribosomal subunit by attaching a phosphate, adenylyl or acetyl radical to the aminoglycoside molecule (87). Although active efflux and target modification (88) are less common, they do remain important in conferring resistance of *P. aeruginosa* to aminoglycosides.

Resistance to fluoroquinolones is the result of structural changes in DNA gyrase and active efflux (89). Mutated DNA gyrase has a low binding affinity for the quinolone

molecules. Fluoroquinolones are also universal substrates for most all efflux systems present in *P. aeruginosa*.

Acinetobacter

Microbiology

The *Acinetobacter* genus comprises Gram-negative, non-fermenting, non-motile, catalase-positive, oxidase-negative, strictly aerobic bacteria. *Acinetobacter* species are considered ubiquitous organisms (90, 91). *A.baumannii* is the most important nosocomial *Acinetobacter* species. It has a low carrier rate and is found rarely (0.5%-3%) on human skin (92, 93).

Disease

In a large surveillance study in the United States, it was found that between 5-10% of cases of ICU-acquired pneumonia were due to *A. baumannii* (94). In addition to being identified as the causative agent in hospital-acquired pneumonia, *A. baumannii* has also been implicated in community-acquired pneumonia, described in tropical regions of Australia and Asia (95, 96, 97, 98, 99). Diagnosis of community acquired *A. baumannii* pneumonia occurs most commonly in people with a history of alcohol abuse and some admission to the ICU (95) and is characterized by secondary bloodstream infection and a mortality rate of 40-60% (99). In addition to pneumonia, *A. baumannii* can also cause serious bloodstream infections. In a US study of blood stream infections from 1995-2002, *A. baumannii* was the 10th most common etiologic agent (100). Sources of *A.*

baumannii blood stream infections are typically line related or attributed to underlying pneumonia, urinary tract (UTI) or wound infection (101). In patients with nosocomial postsurgical meningitis, *A. baumannii* mortality is as high as 70% (102). Endocarditis caused by *Acinetobacter* is not very common (103, 104, 105, 106) and usually involves prosthetic valves. In addition, *Acinetobacter*-caused dophthalmitis or keratitis have been reported in relation to contact lens usage or eye surgery (107, 108).

Pathogenesis

Although there are no known diffusible toxins or cytolysins associated with *A. baumannii*, genes have been identified that are involved in pillus biogenesis, iron uptake and metabolism, QS and the type IV secretion system (109, 110). *A.baumannii* is able to form biofilms, which contributes to drug resistance and evasion of the host immune system (111, 112). *A.baumannii* also often display pilli or fimbriae which are important for adhesion leading to eukaryotic cell invasion and promotion of apoptosis of the eukaryotic cell (113).

Mechanisms of Antibiotic Resistance

In addition to innate resistance mechanisms, *A. baumannii* has acquired many foreign resistance determinants which has lead to a broad resistance profile. The most prevalent resistance mechanism to β -lactam antibiotics is enzymatic degradation. All *A. baumannii* have chromosomally encoded AmpC cephalosporinases (114, 115, 116, 117). In many Gram-negatives this is inducible, however, in *A. baumannii* the over expression

of AmpC is controlled by an upstream IS element known as IS*AbaI* (118, 119, 120, 121, 117).

To resist the effects of carbapenems, *A. baumannii* produces β -lactamases with carbapenemase activity and serine oxacillinases known as Ambler class D OXA type. The enzymes encoded are class D meaning that the products have a higher affinity for imipenem than for meropenem (122, 123).

A. baumannii also expresses MBLs, an Ambler class B-type enzyme (124, 125). MBLs are less commonly identified in *A. baumannii* but are 100-1000 fold more potent than OXA (124). MBLs have hydrolytic activity toward all β -lactams except monobactam and aztreonam. There are 5 currently described MBL groups in the literature (126) with only 3 of these currently identified in *A. baumannii*.

In addition to enzymatic mechanism of resistance to β -lactams, *A. baumannii* can possess many non-enzymatic resistance mechanisms including changes to the outer membrane protein (114, 127, 128, 129), multi-drug efflux pumps (120, 130, 131) and alteration in the affinity or expression of penicillin-binding proteins (132, 133, 134).

Genes coding for all classes (acetyltransferases, nucleotidyltransferases, and phosphotransferases) of AMEs have been identified in *A. baumannii* (135, 115). In addition, a plasmid borne gene, *armA*, impairs the ability of aminoglycosides to bind to 16s rRNA through methylation. Resistance to quinolones appears in the form of described mutations in the *gyrA* and *parC* genes which lead to modifications to DNA gyrase or topoisomerase IV and cause interference with target binding (136, 137). Quinolones are substrates for AdeABC (130, 131) and the MATE pump, AdeM (138). Resistance to tetracyclines and glycylcyclines in *A. baumannii* is the result of efflux

through tet-a and tet-b (139, 140, 141) and the AdeABC pump (131). In addition, *A. baumannii* can employ ribosomal protection to resist the effects of tetracyclines mediated by *tet*(M) and *tet*(O) determinants. Recent reports have demonstrated *in vitro* resistance of *A. baumannii* to polymyxins, but the mechanism remains unknown (141, 142). Trimethoprim and sulfamethoxazole resistance in *A. baumannii* is becoming prevalent in many areas (143). Recently, genes encoding for resistance to trimethoprim (*dhfr*) and resistance to chloramphenicol (*cat*) have been reported (144).

Escherichia coli

Microbiology

E. coli is a rod-shaped, Gram-negative, non-sporulating facultative anaerobe that grows on a variety of substrates, and is a member of the normal flora in mammals (145). Its optimal growth occurs at 37°C, but some laboratory strains can grow in temperatures up to 49°C (146).

Disease

Normally, *E. coli* doesn't cause disease and co-exists with its host for a lifetime, however, there are 3 general clinical syndromes that can result from infection with a virulent *E. coli*: sepsis/meningitis, enteric/diarrheal disease, and urinary tract infections (UTIs) (145).

Pathotypes and pathogenesis

Enteropathogenic *E. coli* (EPEC)

EPEC was the first pathotype of *E. coli* to be described. Currently, EPEC is an important cause of fatal infant diarrhea in developing countries (147). The TTSS is activated in EPEC following binding to epithelial cells. Following activation, effector proteins including Tir, and numerous other molecules are translocated into the host cell. EPEC will then bind to the host cell through an interaction of intimin with Tir. Following this interaction there is activation of protein kinase C (PKC), myosin light-chain kinase, phospholipase C γ and mitogen-activated protein (MAP) kinases which lead to nuclear factor kappa B (NF- κ B) activation and IL-8 production (147, 148, 149). Diarrhea due to EPEC results from multiple mechanisms including inflammation, ion secretion and increased intestinal permeability (145).

Enterohaemorrhagic *E. coli* (EHEC)

EHEC is infective at a very low dose (<100 cells) and causes bloody and non-bloody diarrhea and hemolytic uremic syndrome (HUS) (145). The main virulence factor of EHEC is Stx or verocytotoxin (VT), which in addition to local damage to the colon, damages the renal endothelial cells of the kidneys. Stx is produced in the colon and travels in the bloodstream to the kidneys where it binds to the target cell surface and cleaves ribosomal RNA leading to loss in protein synthesis (150). This leads to renal inflammation (151) through cytokine and chemokine production and this damage leads

to HUS. Besides Stx, most EHEC strains have a type III secretion system and other effector proteins present in EPEC.

Enterotoxigenic *E. coli* (ETEC)

ETEC is the main cause of diarrhea in travelers in developing countries and is an important cause of childhood diarrhea in the developing world (147). ETEC attaches to and colonizes the surface of the mucosa of the small bowel and secretes heat-labile (LT) and heat-stable (STs) enterotoxin. LT causes a permanent activation of adenylate cyclase through its ADP-ribosyl transferase activity leading to increased levels of intracellular cAMP. The increased levels of cAMP induce activation of cAMP-dependant kinases and eventual activation of the CF trans-membrane conductance regulator (CTFR).

CTFR is the main chloride (Cl⁻) channel in epithelial cells and the result of its phosphorylation is increased Cl⁻ secretion and diarrhea (152). LT also causes an increase in secretion of water and mucous as well as an inhibition of absorption through stimulation of prostaglandin synthesis and the enteric nervous system (152).

STs have two sub-classes: STa and STb, of which only the STa class has been associated with disease in humans (147). When STa binds to its guanylate cyclase receptor it stimulates its activity leading to increased intracellular cGMP. The increased intracellular cGMP activates kinases, which increase secretion of water and mucous.

Enteroaggregative *E. coli* (EAEC)

EAEC is the cause of persistent diarrhea in both children and adults and is characterized by its autoaggregative adherence pattern in which bacteria adhere to each

other in a 'stacked brick' pattern (147). EAEC colonizes the intestinal mucosa of the colon causing secretion of enterotoxins and cytotoxins (153) and induces significant mucosal damage (154). There are several toxins and virulence factors attributed to EAEC. Although no single factor has been directly proven, epidemiological studies have implicated similar virulence factors of other enteric pathogens (155, 156).

Enteroinvasive *E. coli* (EIEC)

EIEC pathogenesis is characterized by dissemination into epithelial cells by cell penetration, lysis of the endocytic vacuole and intracellular multiplication followed by movement through the cytoplasm and extension into adjacent epithelial cells (145). It has been shown that EIEC can induce apoptosis in infected macrophages (157). In addition to the type III secretion system that is essential for the invasiveness of EIEC (158), additional virulence factors such as the serine protease Sep A and the aerobactin iron-acquisition system have been described.

Diffusely adherent *E. coli* (DAEC)

DAEC are the cause of diarrhea in children older than 12 months of age (147). The cytopathic effect induced by DAEC strains is distinguished by long cellular extensions that wrap around the adherent bacteria (159).

Uropathogenic *E. coli* (UPEC)

There is a small number of O serogroups of *E. coli* that cause uncomplicated cystitis and acute pyelonephritis and this subset of *E. coli* is distinct from the commensal

E. coli strains found in the lower colon. There is no single phenotypic profile that causes UTIs but specific adhesins and fimbriae aid in colonization (160, 161). In addition, several toxins attributed to UPEC have been described, including cytotoxic necrotizing factor, haemolysin and the auto transported protease Sat (161).

Meningitis/sepsis-associated *E. coli* (MNEC)

MNEC is the most common cause of Gram-negative neonatal meningitis (162, 163). Strains that cause meningitis are usually of the K1 capsule type and comprise only a small number of O serogroups.

Mechanisms of antibiotic resistance

E. coli is often resistant to β -lactam antibiotics, this resistance is usually mediated by one or more β -lactamases enzymes (74, 164). Ampicillin resistance is usually mediated by the OXA type of β -lactamases (79, 164). Quinolone resistance is most often present in the form of alteration of the target of the quinolone molecule, DNA gyrase. Aminoglycoside resistance is most often in the form of aminoglycoside modifying enzymes (165, 166).

Klebsiella pneumoniae

Microbiology

K. pneumoniae is a Gram-negative, non-motile, facultative anaerobic, encapsulated lactose fermenting bacteria. The primary reservoir for *K. pneumoniae* is

humans, although it can be found in soil and water (167, 168, 169). Carrier rates in humans ranges from 1 to 6 percent in the nasopharynx (although, higher rates have been seen in alcoholic patients), 5-38 percent in stools, and is rarely carried on skin (170). These carrier rates, however, are dramatically increased in hospitalized patients with 77, 19 and 42 percent carrier rates in the stool, pharynx and hands, respectively (170). These higher rates of carriage are related to the use of antibiotics (170, 171).

Disease

K. pneumoniae is most often described in patients with occupational exposure (often in the paper industry) (172) or as a nosocomial pathogen in patients with impaired host defenses such as alcoholism, malignancy, chronic obstructive pulmonary disease, glucocorticoid therapy, renal failure, diabetes mellitus, and hepatobiliary disease (173, 174, 175, 176, 177, 178). In the nosocomial setting it has been associated with urinary and pulmonary tract infection, and bacteremia. *K. pneumoniae* causes, although less commonly, community-acquired infection pneumonia, UTI, and a liver abscess syndrome (primarily in east Asia) that has been associated with metastatic infections.

Pathogenesis

The pathogenesis of infection with *K. pneumoniae* is mediated by 5 major virulence factors: i) capsular serotype, ii) hypermucoviscosity, iii) lipopolysaccharide, iv) siderophores and v) pili (170). There are 77 different serotypes of capsular polysaccharide antigens of *K. pneumoniae*, varying worldwide by region (179, 180, 181, 182, 183, 184). In human urine and sputum clinical isolates the most common serotype

for capsular antigen is the K2 serotype (179). It has been demonstrated that the most common serotypes in Europe and North America are 2, 21 and 55 (179). In contrast, the most common serotype in liver abscess, bacteremia and septic endophthalmitis in Taiwan is K1 (178, 180-182, 183) and the most common serotype in Australia from urinary, sputum and blood isolates is K54 (183). The K1 and K2 capsular polysaccharide antigen expressing isolates have been shown to have a higher lethality when administered to mice than the non-K1/K2 isolates (185). The 2 major reasons for increased virulence of the K1/K2 serotype isolates are: i) these serotypes are more often hypermucoviscous than non K1/K2 strains and ii) the lack of mannose or rh-mannose sequences that protect the isolates from lectin mediated phagocytosis (186).

Isolates that have the hypermucoviscosity phenotype in *K. pneumoniae* are more resistant to complement-mediated serum killing than those lacking this phenotype (187, 188). Hypermucoviscosity has been shown to correlate to a higher rate of invasive infection when compared to non-invasive strains (187). In addition, many clinical studies have shown a direct association between destructive tissue abscess syndrome and the hypermucoviscosity phenotype (181, 189, 190, 191).

The lipopolysaccharide O side chain can inhibit C1q and C3b in serum from binding to the *K. pneumoniae* cell membrane rendering it immune to complement-mediated membrane damage (192, 193). *K. pneumoniae* isolates that lack the lipopolysaccharide O side chain have been shown to have increased sensitivity to complement-mediated killing (192, 188, 193). In addition to inhibiting complement-mediated killing, *K. pneumoniae* lipopolysaccharide can trigger cytokine pathways by

acting as an endotoxin leading to septic shock and an increase in the propensity for bacteremia (194).

Synthesis of siderophores is important to many bacteria, including *K. pneumoniae*, due to the low levels of iron present in the microenvironment of an infection (195). The two main siderophores produced in *K. pneumoniae* are enterobactin, which is synthesized by most all strains, and aerobactin, which is found in less than 10 percent of strains (196, 197, 198). *K. pneumoniae* strains that produce aerobactin are more virulent than those that do not, and it has been shown that the transfer of the aerobactin gene into a non-virulent *K. pneumoniae* strain can enhance virulence by 100-fold (199).

K. pneumoniae expresses 2 different types of pili, named type 1 and type 3 (200). Type-1 pili are found in all members of the Enterobacteriaceae and mediate adherence to epithelial cells (201). The gene encoding these pili (*fimH*) is present in more than 98% of *K. pneumoniae* (189, 202). The type 3 pili contribute to virulence in *K. pneumoniae* by attaching to epithelial cells (203) leading to colonization and proliferation followed by infection. In addition, type 3 pili are a requirement for *K. pneumoniae* to form biofilms (204).

Mechanisms of antibiotic resistance

Drug resistance in *K. pneumoniae* is due to expression of chromosomal AmpC β -lactamases that are constitutive and inducible, as well as plasmid-encoded ESBLs and carbapenemases.

Enterococci

Microbiology

Enterococci are a Gram-positive facultative anaerobic organism that grows in chains and are distinguished by 4 criteria: i) react with group D antiserum, ii) demonstrate pyrrolidonyl arylamidase and leucine aminopeptidase, iii) grow in 6.5% sodium chloride and iv) hydrolyze esculin in the presence of bile. Enterococci are a part of the normal intestinal flora and have recently become an issue as a multi resistant pathogen in the hospital setting (205, 206).

Disease

Nosocomially, the most common infections due to enterococci are UTIs followed by intra-abdominal and pelvic infections (207). Enterococci are also the causative agent in surgical wound infections, neonatal sepsis, meningitis, bacteremia, and endocarditis (208).

Pathogenesis

Several studies have identified virulence factors in enterococci, the most important and prevalent being aggregation substance (AS), surface protein, gelatinase and *fsr* two-component system, pili, MSCRAMMs (microbial surface components recognizing adhesive matrix molecules), CW and capsular polysaccharides, and glycolipids. AS are a group of surface proteins that direct bacterial aggregation and whose expression is induced by a peptide pheromone (209, 210, 211). In addition to

bacterial aggregation, AS have been reported to facilitate bacterial internalization (212, 213, 214). An enterococcal surface protein of great interest is the product of the gene *esp* (215, 216). Proteins encoded by the *esp* gene have been identified as factors involved in colonization of the urinary tract (217). Gelatinase is a secreted bacterial protease controlled by the *fsr* two-component system and has been shown to have an important role in biofilm formation (218). The pilli of enterococci facilitate biofilm formation and adhesion, contributing to the pathogenesis of biofilm-associated diseases such as endocarditis (219) and UTIs (220, 221). MSCRAMMs contribute to pathogenesis of enterococci by interacting with the extracellular matrix leading to colonization of human tissues. The CW of enterococci is important for virulence because it prevents complement activation and killing by phagocytes through evasion of the host's immune system (222). Glycolipids play an important role in virulence and are involved in forming a permeable layer between the cytoplasm of the host cell and the environment (223, 224).

Mechanisms of antibiotic resistance

Enterococci have an intrinsic resistance to low levels of aminoglycosides and clindamycin, nalidixic acid, lincosamides, low levels of penicillinase susceptible penicillin, penicillinase resistant penicillins and cephalosporins (225). Because their penicillin binding proteins (PBP's) are low affinity, the enterococci retain their ability to synthesize the CW in the presence of these antibiotics (225). In addition to intrinsic resistance to β -lactams, enterococci can become tolerant to penicillin in as few as 5 doses (207) and can produce β -lactamase enzymes (225-227, 228). Low-level resistance

to aminoglycosides is a trait shared by all enterococci and is attributed to low uptake of these molecules (225). Enterococci can acquire high level aminoglycoside resistance through the expression of AMEs that are transferable from one organism to another the most common of which are (i) dual function 2'phosphotransferase and 6'acetyl transferase that confer resistance to all aminoglycosides except streptomycin, (ii) 3'phosphotransferase which confers resistance to kanamycin and penicillin-amikacin, (iii) 6'adenyl transferase which inactivates streptomycin (225). An important clinical problem is the rapid spread of enterococci that have also become resistance to glycopeptides (229, 230-232) following the first report of vancomycin-resistant enterococci (VRE) in the 1980s (233). Most commonly resistance to glycopeptides in enterococci has been reported in 3 phenotypes i) Van A which is inducible and confers high level resistance to vancomycin and teicoplanin, ii) Van B which confers varied levels of resistance to vancomycin and is sensitive to teicoplanin and iii) Van C which confers constitutive low level resistance to vancomycin and sensitivity to teicoplanin (234, 235).

Staphylococcus aureus

Microbiology

S. aureus is Gram-positive, catalase and coagulase positive, mannitol fermenting, non-motile common commensal organism found in 30-50% of healthy humans. *S. aureus* is an aerobic organism but can tolerate growth at low oxygen, under high osmotic pressure and at a wide temperature range (236).

Disease

Although *S. aureus* is a common commensal, it is also an opportunistic pathogen that can cause many diseases in humans and other species. Infections caused by *S. aureus* range from mild skin diseases to invasive illness (236). Prolonged infections lead to development of osteomyelitis or necrotizing fasciitis (237, 238). *S. aureus* sepsis is usually associated with bacteremia, and can be secondary to bacterial infection of the urinary tract, bone, kidney, or lungs (239, 240). *S. aureus* is a causative agent of toxic shock syndrome (TSS) and gastroenteritis (241). In addition, *S. aureus* is the most frequently isolated pathogen from patients suffering from ventilator-associated pneumonia (242).

Pathogenesis

S. aureus possesses both secreted and structural products that contribute to its virulence and pathogenesis. It has a variety of surface attachment-improving agents known as MSCRAMMs that mediate attachment to host cells and therefore initiate colonization. *S. aureus* exotoxins and super antigen toxins have been implicated in gastroenteritis and TSS (243).

Mechanisms of antibiotic resistance

Methicillin resistant *S. aureus* (MRSA) accounts for the majority of nosocomial infections (244). Nosocomial strains of methicillin resistant *S. aureus* known as

hospital-associated *S. aureus* (HA-MRSA) infects those that have established risk factors such as prolonged hospitalization or antimicrobial therapy, surgical procedures, dialysis, indwelling vascular devices, and proximity to patients in the hospital who are infected or colonized with MRSA (236). HA-MRSA strains are usually resistant not only to methicillin but also to many classes of antibiotics (245). MRSA infecting individuals outside of the healthcare facilities is referred to as community-associated MRSA (CA-MRSA) (246, 247). Contrary to HA-MRSA, CA-MRSA strains are mostly resistant to β -lactams (248). Although CA-MRSA is traditionally less drug resistant, clusters of disease of a multidrug-resistant USA300 isolates that contain multiple resistance genes, rendering it resistant to β -lactams, fluoroquinolones, tetracycline, macrolide, clindamycin, and mupirocin, surfaced in the community (249, 250).

Daptomycin is a lipopeptide antibiotic that was approved by the United States Food and Drug Administration (FDA) in 2003 for treatment of complicated skin and soft tissue infections caused by bacteria, including methicillin-resistant *Staphylococcus aureus* (Cubist Pharmaceuticals, Inc. 2003. (Daptomycin (Cubicin) package literature, Cubist Pharmaceuticals, Inc., Lexington, Mass.). However, treatment failure due to daptomycin resistance has been recently reported (251).

Antimicrobial peptides (AMPs)

AMPs are short amino acid sequences derived from larger precursors. They are present in many organisms ranging from prokaryotes to humans. These peptides have an ancient lineage and are widespread throughout the plant and animal kingdoms, which

suggests that they have played an integral part in the evolution of multicellular organisms. These peptides are used as potent broad-spectrum antimicrobial agents with a diverse range of targets; the presence of a lipid membrane being the only common characteristic among their targets (252, 253, 254). AMPs are one of the earliest developed molecular effectors of innate immunity, and beyond antimicrobial effects, these peptides have been shown to confer many functions in wound healing (255), chemotaxis (256), and the induction of adaptive immune responses to foreign antigens (257). Almost all antimicrobial peptides share a common composition consisting of hydrophobic and hydrophilic cationic amino acids that confer an amphipathic structure (258).

Amphipathic alpha helices have been reported in a variety of proteins (259) and are defined by their opposing polar and non-polar faces oriented along a helical axis. These helices make up 50% of the alpha helices found in soluble globular proteins (260, 261). There are a variety of purposeful characteristics attributed to this class of helices, such as lipid association, membrane perturbation, hormone-receptor catalysis, transmembrane signal transduction, regulation of kinase-calmodulin signal transduction and transmembrane helical bundle formation (259).

AMPs are divided into 5 major groups according to their amino acid composition and structure (262, 263, 261). The first class of AMPs known as the anionic peptides are small peptides present in surfactant extracts, airway epithelial cells and bronchoalveolar lavage fluid (261, 264, 265). These anionic peptides require zinc as a cofactor and are active against both Gram-negative and Gram-positive bacteria. Cationic peptides are enriched for a specific amino acid. These peptides lack cysteines and are mostly linear

although some form extended coils. There is also a subgroup of anionic and cationic peptides that contain cysteines and form disulphide bonds and stable β -sheets. This group of AMPs covers human and insect defensins. An additional subgroup includes anionic and cationic peptides that are fragments of larger proteins. These are similar in structure and composition to the other classes of antimicrobial peptides but their role in innate immunity is not clear. The final class of antimicrobial peptides are the linear cationic α -helical peptides, which usually contains less than 40 amino acids and can have a hinge or 'kink' in the middle (266, 267). When in an aqueous environment, these peptides are highly disordered; however, when in the presence of SDS micelles, lipid A, phospholipid vesicles and liposomes, these peptides will convert into an alpha helix (262).

The mechanism of action of amphipathic AMPs has been fairly well defined (268). Three main steps must take place for AMP-mediated cell killing to occur: (i) attraction of the peptide to the cell surface, (ii) attachment of the peptide to the cytoplasmic membrane and (iii) peptide insertion followed by membrane permeabilization. There are 6 main characteristics that affect antimicrobial activity and specificity: size, sequence, charge, conformation and structure, hydrophobicity and amphipathicity. Currently, there are three widely accepted models of peptide insertion and permeabilization of the membrane (Fig. 1-3, (268)). The carpet model describes the parallel attachment of the peptides to the surface of the lipid bilayer where the anionic phospholipid head groups on the outer surface of the membrane form a thick layer or 'carpet' (269). At high concentrations these peptides are proposed to disrupt the membrane, similar to detergent forming micelles (270, 271), leading to the loss of

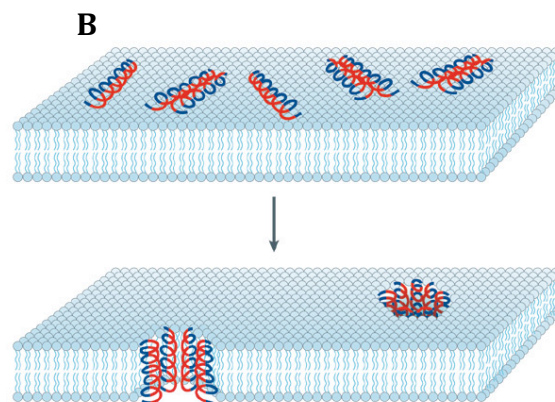
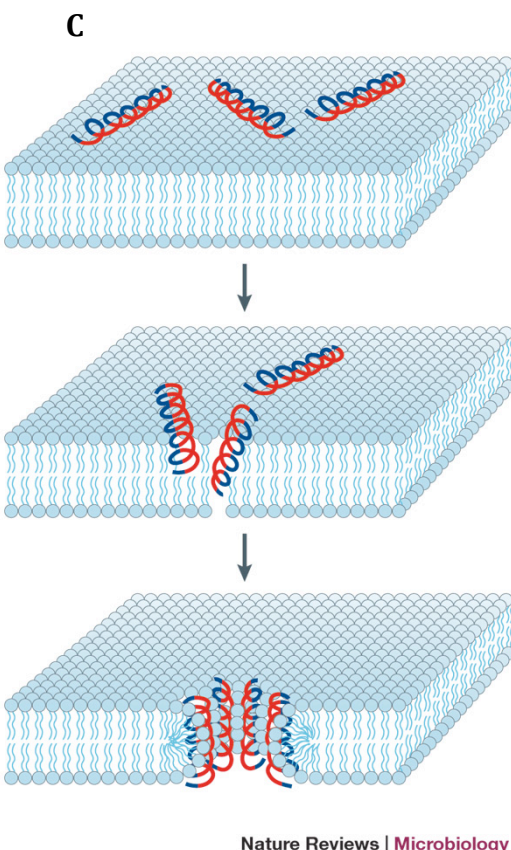
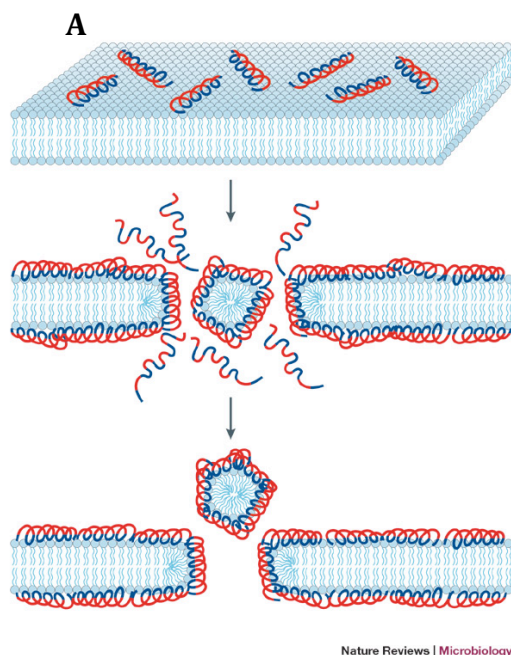


Figure 1-3. Modes of action of antimicrobial peptides.

(A) Carpet model.

“In this model, the peptides disrupt the membrane by orienting parallel to the surface of the lipid bilayer and forming an extensive layer or carpet. Hydrophilic regions of the peptide are shown coloured red, hydrophobic regions of the peptide are shown coloured blue”

(B) Barrel stave model.

“In this model, the attached peptides aggregate and insert into the membrane bilayer so that the hydrophobic peptide regions align with the lipid core region and the hydrophilic peptide regions form the interior region of the pore. Hydrophilic regions of the peptide are shown coloured red, hydrophobic regions of the peptide are shown coloured blue.”

(C) Torodial pore model.

“In this model the attached peptides aggregate and induce the lipid monolayers to bend continuously through the pore so that the water core is lined by both the inserted peptides and the lipid head groups. Hydrophilic regions of the peptide are shown coloured red, hydrophobic regions of the peptide are shown coloured blue.”

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membrane integrity (Figure 1-3A; (268)). The barrel stave model (Figure 1-3 B, (268)) consists of the insertion and aggregation of the peptides in the lipid bilayer. The aggregation results in the formation of a pore where the hydrophilic regions of the peptide lies in the inner core of the aperture (268). The third proposed mechanism of action is the toroidal pore model (Figure 1-3C, (268)). In this model, the peptide attaches to the surface of the membrane, aggregates and induces bending in the lipids so that the interior of the pore is lined with the lipid head groups and the inserted peptide (272). Although these three theoretical models seem distinct, it has been proposed that they are likely related and peptide concentration (273).

Modified peptides in current use and testing

There are many examples of modified host defense peptides and synthetic AMPs that have entered into human testing (274, 275). Currently, there are 12-15 in different stages of clinical development (275) however, the only peptide antibiotics currently used are Polymyxin B/E, Gramicidin S and daptomycin. Unfortunately, both the polymyxins and Gramicidin S are only used topically and not used systemically because they have been shown to be extremely toxic (275). There has been an effort to modify both peptides to make them less toxic and although it has not been successful for Gramicidin S there is a modified form of Polymyxin that is currently used systemically for lung infections of CF patients (275). The FDA, however, approved Daptomycin, in 2003 for the treatment of skin and skin-suture infections caused by susceptible Gram-positive organisms.

Daptomycin

Daptomycin is a 13-member amino acid cyclic lipopeptide with a decanoyl side-chain (276) that has bactericidal activity against Gram-positive organisms. Daptomycin is produced by *Streptomyces roseosporus* as a fermentation product (277-280, 281). The proposed mechanism of action of Daptomycin is identical to that of antimicrobial peptides described previously, insertion into the bacterial cell membrane causing depolarization, although the proposed method of cellular death is arrest of DNA, RNA and protein synthesis (276, 282), not cell lysis.

KLA peptides

One type of peptide that has been thoroughly studied is a group of α -helical amphipathic peptides derived from the model peptide KLALKLALKALKAALKLA-NH₂ originally used for studying the interactions between peptides and lipid interfaces (283, 273, 284, 285, 286, 287, 288). These derivatives are collectively called the KLA-derived peptides.

(KLAKLAK)₁₋₃ is a KLA-peptide that was originally tested for its ability to kill both eukaryotic and prokaryotic cells by the McLaughlin group in 1996 (289). Jadvapoor et al. designed several different KLA-peptides to determine the effect of length, sequence, and structure on biological activity. It was found that all of the leucine- and alanine-containing peptides of the same length had similar MIC values. In addition, it was shown that the 7-mers were inactive and the leucine-containing 21-mers were 2 times as potent at killing bacteria as the 14-mers, while maintaining low mammalian-cell toxicity (289).

Obstacles for clinical use of antimicrobial peptides

The major issues for clinical use of AMPs as antibiotics are the same issues faced with development of most forms of antibiotics: (i) high cost of commercial scale synthesis, (ii) toxicity, (iii) proteolytic degradation/inactivation, (iv) resistance and (v) poor distribution in the body. The first issue, cost of synthesis, is important to consider because production costs for peptides is generally higher than the cost to produce 'conventional' antibiotics. Toxicity is important to consider because for an antibiotic to be effective it must be highly selective towards bacterial cells while having minimal toxicity against eukaryotic cells. Often AMPs have considerable hemolytic activity (290) which hinders their use as systemic antimicrobials, and restricts their use to topical applications. Development of peptides that have a reduced toxicity and enhanced antimicrobial activity is possible with the understanding of features that lead to selectivity of the peptide. Proteolytic degradation of AMPs is a common problem, with most peptides being degraded, or bound by proteins in the blood stream and therefore rendered inactive. Commonly, peptide structures are modified so that they are no longer recognized or degraded by proteolytic enzymes (291). This approach has been successful with a number of peptides including magainin analogues (292) where tyrosines are substituted with β -tyrosines that are shortened by one carbon unit in the side chain. The substitution allows the peptide to have the same structure as the naturally occurring peptide as well as have a greater stability. It can be assumed that if peptide interaction with bacterial membranes leading to lysis of bacterial cells is membrane receptor dependant, then that receptor would require a specific chirality of the peptide to be functional. However, it has been shown that the enantiomeric forms of

AMPs with all D-amino acids have equal activities when compared to their L counterparts (293, 294, 295). Additionally, D-form AMPs have a higher stability against proteolytic degradation (296, 297).

The issue of resistance is not as serious of a concern with AMPs because it is assumed that resistant strains will not easily arise because the target of most AMPs is the bacterial membrane, a structure that it is essential for the bacterium's survival. This assumption is strengthened by the observation that AMPs have been found in organisms for millions of years with only a few incidences of resistance ever demonstrated.

Poor distribution of AMPs this can be overcome with site-specific administration and targeting of the peptides.

Significance of this research

Currently, it is estimated by the National Institute of Allergy and Infectious Disease that over 70% of hospital-acquired infections are resistant to at least one of the antibiotics used to treat them. Routine use of antimicrobial agents has reduced the threat posed by infectious diseases and--along with improvements in sanitation, housing, nutrition, and wide-spread immunization programs--has led to a marked reduction in deaths from diseases that were previously widespread, untreatable, and frequently fatal. However, the overuse and misuse of these antimicrobial agents has led to an increase in resistant organisms, which have spread through many hospitals and communities. According to NIH estimates, between 5-10% of all hospital patients develop an infection, leading to an increase of about \$5 billion in annual U.S. healthcare costs.

The rise of multi-drug resistance represents a clear need for new, novel antibiotics to combat these resistant infections; however, antimicrobial research and development of new antibiotics is lagging far behind the ability of organisms' ability to acquire resistance. In the 1930s and 1940s there were 4 new classes of antibiotics introduced: sulphonamides, β -lactams, aminoglycosides and chloramphenicol. Following these developments in the 1950s and 1960s there were 6 more classes of antibiotics that were developed and approved: tetracycline, macrolides, glycopeptides, rifamycins, quinolones and Trimethoprim. Following 4 decades of initial discovery, from the 1970s to the 1990s there were no new antimicrobial classes approved, and since 2000 there has only been approval for a few new classes of antimicrobials: oxazolidinones, cyclic lipopeptides and glycylcyclines, all of which are approved for the treatment of Gram-positive pathogens. The time required to take an antibiotic from its discovery to market is around 8-12 years (298). Although there is a limited number of antimicrobial agents recently approved or in the late stages of development, these are directed solely toward Gram-positive pathogens leaving a lack of antibiotics in the development stages to take on the present problem of multi-resistant Gram-negative pathogens (299, 300). The current options to treat MDR infections are becoming limited as these bacteria expand their ability to evade existing antibiotics by developing of various resistance mechanisms. Organisms of great concern due to the ability to become multi-resistant are *P. aeruginosa*, *K. pneumoniae*, *E. coli*, *E. faecalis*, *A. baumannii* and *S. aureus*.

In this study we have shown that a synthetic AMP able to disrupt bacterial membrane non-specifically is active against Gram-negative organisms such as *P.*

aeruginosa, *K. pneumoniae*, *E. coli* and *A. baumannii*, regardless of their antibiotic resistance profiles. Although the scientific community does not see development of resistance to AMPs as a concern, reduced sensitivity of *S. aureus* and *E. faecalis* to de-novo AMP, DAP, emerged as a result of treatment. Resistance associated with DAP include, most importantly, modifications to the thick peptidoglycan layer surrounding the CM of Gram-positive organisms. Since this is a Gram-positive specific cell feature, the mechanism of resistance related to changes in the peptidoglycan layer is also specific to Gram-positive cells.

Chapter II

Materials and Methods

Chapter II

Materials and Methods

Media and growth conditions:

Gram-negative bacterial isolates. *P. aeruginosa* 10145, *A. baumannii* 19606 and *E. coli* strain 25922 were obtained from ATCC. *Pseudomonas aeruginosa* PAO1 and PA14 were provided by Dr. Frederick M. Ausubel, Harvard University. Gram-negative clinical isolates were provided by the Clinical Microbiology Laboratory at St. Lukes Episcopal Hospital. All strains were grown at 37°C in cation-adjusted Mueller Hinton broth (CA-MHB) unless otherwise stated. The biofilm inhibition assays were performed in CA-MHB and 10% FBS-RPMI.

***E. faecalis* bacterial isolates.** The two vancomycin-resistant *E. faecalis* isolates used were recovered from the bloodstream of a 64-year-old woman (301). The first isolate (daptomycin-susceptible) was recovered after the administration of linezolid and ciprofloxacin (daptomycin MIC, 1 µg/ml). The second isolate (daptomycin MIC 16 µg/ml) was obtained from the blood of the same patient two weeks after the start of daptomycin and amikacin therapy. The daptomycin resistant isolate exhibited the same pulsed field gel electrophoresis pattern and had identical susceptibilities to antibiotics as the parental susceptible isolate, except for daptomycin (301). Both isolates were grown in brain heart infusion broth.

α (KLAKLAK)₂ was obtained from Poly-Peptide-Labs.

Stability assays. The stability of $_D(KLAKLAK)_2$ compared to $_L(KLAKLAK)_2$ in the presence of human liver microsomes was determined by mass spectrometry. Briefly, 5 μ l of human liver microsomes (pooled from multiple donors; BD Biosciences) were diluted in 75 μ l of 50 mM potassium phosphate buffer pH 7.4. $_D(KLAKLAK)_2$ and $_L(KLAKLAK)_2$ were added to the mixture (10 μ l of a 10 mg/ml solution in water) and warmed for 5 minutes at 37°C. The reaction was started with the addition of 10 μ l of 20 mM NADPH in phosphate buffer. Aliquots (10 μ l) were removed pre- and post-addition of NADPH at 1, 10, 30 and 60 minutes and 24 hours. The reactions were stopped with 5 μ l of 100% trifluoroacetic acid. All samples were frozen at -80°C until processed by matrix assisted laser desorption/ionization time-of-flight (MALDI) with double time-of-flight (TOF²) detection.

MALDI-TOF² mass spectroscopy. The samples were centrifuged at 13,200 rpm for 30 minutes at 4°C to remove liver microsomal debris and then 2 μ l of each sample was added to 198 μ l of 0.1% TFA solution. Samples were then further diluted 1:10 in α -cyano-4-hydroxyl cinnamic acid (α CHCA; 10 mg/ml in 50% acetonitrile 0.5% TFA). Each sample was loaded to 3 spots on a MALDI-TOF plate and MS data acquired on each spot in duplicate. ^{13}C - $_D(KLAKLAK)_2$ was used as the internal standard for quantification. Quantification was performed by integrating the peak area of individual spectra and was graphically displayed relative to the internal control.

Susceptibility assays (MIC). Antimicrobial susceptibility testing was performed as recommended by the NCCLS (CLSI document M100-S16 CLSI). All bacterial isolates

were tested by broth micro dilution in CA-MHB. After incubation for 18h at 37°C, the MIC is defined as the lowest concentration of the antimicrobial agent that inhibits visible growth of the tested isolate as observed with the unaided eye. All experiments were performed in triplicate.

Time-kill curves. Overnight bacterial cultures were diluted in fresh medium, and allowed to reach exponential phase of growth. Increasing concentrations of peptide and/or antibiotic were added and the cultures were incubated at 37°C with shaking for up to 24 hours. To determine viable bacteria, aliquots were taken at 4, 10 and 24 h after treatment and dilutions were plated onto LB agar. The plates were incubated at 37°C and the colony forming units (CFU) were counted after 24 h. All assays were performed a minimum of 3 times with representative data shown.

Synergistic assays. The synergistic activity between $D(KLAKLAK)_2$ and piperacillin was assayed by time kill curves in CA-MHB as described earlier (302). Briefly, the samples were set up using concentrations of $D(KLAKLAK)_2$ and piperacillin at $\frac{1}{2}$ the MIC, as determined by broth dilution. One sample of each $D(KLAKLAK)_2$ alone, piperacillin alone, $D(KLAKLAK)_2$ and piperacillin together and a control with no antibiotics were assayed.

Biofilm assays. *P. aeruginosa* PAO1 biofilm was grown as described previously (303). Briefly, PAO1 overnight culture was diluted 1:100 in LB or 10% FBS RPMI into 96-well plates and incubated for 24 hrs at 37°C without agitation. To assess the effect of

$D(KLAKLAK)_2$ treatment on the biofilm, increasing concentrations of peptide diluted in LB or 10% FBS RPMI were added to the 24hr-old biofilm. The next day, *P. aeruginosa* biofilm was fixed with 4% paraformaldehyde and stained with 0.1% crystal violet (CV) for 10 min at room temperature. Excess stain was removed with water and the plates were dried overnight at room temperature. CV stain was dissolved in 95% ethanol and the absorbance of a 1:5 dilution in 95% ethanol was measured at 540 nm. All assays were performed a minimum of 3 times with mean of the 3 experiments shown.

Growth phase-dependent activity. The ability of $D(KLAKLAK)_2$ to induce membrane permeability was investigated using the highly sensitive *Baclight* kit (Invitrogen). Overnight cultures of *P. aeruginosa* PAO1 and PA14 were diluted 1:100 in media. Six milliliter aliquots were removed before resuspension (stationary phase), 30 minutes after resuspension (lag phase), 1h after resuspension (early exponential phase) and 2 hours after resuspension (late exponential phase). Bacteria were washed in PBS and treated with increasing concentration $D(KLAKLAK)_2$ or 70% isopropanol. After 1 hour, bacteria were collected, washed and resuspended to OD₆₀₀ of 0.03. An equal mixture of SYTO9 and propidium iodide (3ul) was added to each 1 ml of sample. The samples were incubated 15 minutes at room temperature in the dark. Images were acquired on an Olympus IX71 inverted microscope attached to a Lumen 200 Fluorescence Illumination system with fluorescein and Texas Red bandpass filter sets made by Prior Scientific. Images were collected on the Slidebook 5.0 software.

***P. aeruginosa* electron microscopy.** *P. aeruginosa* was grown to McFarland's standard 0.5 as described above. Bacterial cells were recovered by centrifugation, resuspended in saline and treated with freshly prepared $D(KLAKLAK)_2$ at varying concentrations. The organisms were exposed to $D(KLAKLAK)_2$ for 25 minutes. Bacteria were collected, washed and fixed with a solution containing 3% glutaraldehyde plus 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.3. After fixation, the samples were washed in 0.1 M cacodylate buffer, postfixed in 4% Millipore-filtered potassium permanganate for 1 hour, washed in distilled water and stained en bloc with 1% Millipore-filtered aqueous uranyl acetate for 1 hour. The samples were dehydrated in increasing concentrations of ethanol, three changes in propylene oxide, infiltrated and embedded in LX-112 medium. The samples were polymerized in a 70° C oven for 3 days. Ultrathin sections were cut in a Leica Ultracut microtome (Leica, Deerfield, IL), stained with uranyl acetate and lead citrate in a Leica EM Stainer, and examined in a JEM 1010 transmission electron microscope (JEOL, USA, Inc., Peabody, MA) at an accelerating voltage of 80 kV. Digital images were obtained using the AMT Imaging System (Advantage Microscopy Techniques Corp., Danvers, MA).

***E. faecalis* electron microscopy.** DAP-sensitive and DAP-resistant ultrastructural characteristics were assessed by transmission electron microscopy (TEM). Bacteria were collected, washed and fixed with a 3% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.3. Samples were incubated for 5 min in Millonig's solution followed by a 45 min incubation in 50/50 OsO_4 /Millonig's solution. After 5 min in ddH_2O , samples were step-wise dehydrated using increasing concentrations of ethanol

solutions as follows: 5 min in 50% ethanol, 10 min in 70% ethanol, 10 min in 95% ethanol, washed 3 times for 10 min in 100% ethanol. Dehydrated samples were washed 3 times for 10 min in propylene oxide, followed by incubation in 50% LX-112 for 120 min, then 100% LX-112 for 120 min and were imbedded in BEEM capsules and incubated in a 70° C oven overnight to polymerize. To section the tissue, 500 nm sections were cut from each block using a glass knife on a Leica Ultracut R microtome and were stained with 0.5% Toluidine Blue for 1 minute on a hot plate at 80 °C. Slides were washed and inspected for fitness. The best blocks were then trimmed and thin sections (80 nm & 100 nm) were cut using a DiATOME diamond knife, one each of the two thicknesses were floated on either 100 or 150 mesh copper grids (Electron Microscopy Sciences) and heat fixed in a 70 °C oven for at least one hour. The grids were stained for 15 minutes using 2% uranyl acetate, rinsed with ddH₂O, stained 5 minutes in Renold's lead citrate, rinsed and dried in 70 °C oven. The specimen grids were imaged in a JEOL 1200 transmission electron microscope at 60kV with digital images collected using a 1k X 1k Gatan BioScan camera, Model 792, on a JEOL JEM-1400 cryo-transmission electron microscope. To quantify the number of cell division events (cells with a septum), cells were counted in 5 random consecutive fields in two blinded experiments. Similarly, the average cell wall thickness was determined by measuring a minimum of 3 times per cell on cells in 5 different fields. CW thickness was measured from the outer border of the CM to the outer edge of the CW. Mean (\pm SD) CW thickness was determined for each strain and mean differences compared with a t-test.

Liposome Formation. *E. coli* extract, 1,2-distearoyl-sn-glycero-3-phospho-L-serine (PC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (PE), 1,2-dioleoyl-sn-glycero-3-phosphatidylglycerol (PG), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (PS), cholesterol, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-PE (NBD-PE) and *N*-(rhodamine B sulfonyl)-PE (Rh-PE) were procured from Avanti Polar Lipids (Alabaster, AL). All phospholipids were used without additional purification. The fluorescent dyes were dissolved together with unlabeled lipid. Solvent was evaporated under a steady stream of argon. After the solvent evaporation, lipids were resuspended to a concentration of 1mg/mL in PBS and sonicated for 10 minutes. Subsequently, the preparation was stored at 4°C until use. For the FRET measurements liposomes were made with 5% fluorescent lipid (NBD-PE or Rh-PE) and 95% unlabeled lipid, by weight. For specific lipid interactions, increasing weight percentages of PC, PS, PE, or PG were added to *E. coli* extract. All assays were performed in triplicate with average and standard deviation shown.

Flourescence resonance energy transfer (FRET). To determine the ability of α (KLAKLAK)₂ to disrupt liposomal membranes, increasing peptide concentrations were added to an equal mixture of Rh-labeled and NBD-labeled liposomes. After 24 h, fluorescence transfer was measured using a Spectramax M5 spectrophotometer. The excitation wavelength was 460 nm (excitation wavelength of NBD-PE) and the emission spectrum was measured between 500-620 nm (534 nm emission of NBD-PE and 590 nm emission of Rh-PE) with bandwidths: excitation 9 nm and emission 15 nm.

GUV formation. A thin film (~300 μL) of 1% agarose in deionized water was spread onto a glass slide and then dried at 40°C for 3 hours. A mixture of *E. coli* extract and Rh-PE (30 μl) in chloroform was painted across the agarose film and allowed to dry under vacuum pressure for 2h. Slides were then placed into 150 mM KCL buffer and the agarose was allowed to re-hydrate for a minimum of 1h. Pictures were taken 5 seconds after the addition of $\text{D}(\text{KLAKLAK})_2$. Diluent alone (KCl buffer) was used as negative control. All assays were performed a minimum of 3 times with representative data shown.

Gram-negative membrane potential measurements. Bacteria were collected from a mid-exponential culture, washed in buffer (5mM HEPES pH 7.2, 5mM glucose), and resuspended in the same buffer to $\text{OD}_{600\text{nm}}$ of 0.05. $\text{diSC}_3(5)$ and EDTA were added to the bacterial suspension at final concentrations of 0.4 μM and 0.2 mM EDTA, respectively. The mixture was incubated in the dark for 1h to allow maximal uptake of the $\text{diSC}_3(5)$. The osmotic gradient was equilibrated to a final concentration of 100 mM KCl. Subsequently, the mixture was subjected to increasing concentrations of $\text{D}(\text{KLAKLAK})_2$ in buffer. $\text{diSC}_3(5)$ fluorescence was monitored using a Spectramax M5 spectrophotometer at excitation of 622 nm and emission of 670 nm. Relative fluorescence intensities were normalized to the effect of PBS, which was assigned a value of 0% and 100% isopropanol, which was assigned a value of 100%. All assays were performed a minimum of 3 times with representative data shown.

Gram-negative outer membrane permeability. A 5mL overnight culture of bacteria was used to inoculate 50 mL of LB broth. Mid-log phase bacteria were collected, washed once and resuspended in HEPES buffer (5mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) HEPES pH 7.2, 5 mM sodium azide) to a final concentration OD_{600nm} of 0.5. The bacterial suspension was added to a clean plastic cuvette and the OD_{600nm} was measured for 10 s. Lysozyme was then added to a final concentration of 50 µg/mL and the OD_{600nm} was measured for an additional 10 s. Subsequently, increasing concentrations of $D(KLAKLAK)_2$ in small increments (5-10 µL at a time) were added to the sample. The turbidity of the sample was measured after the lysis process stabilized (as seen by a stabilization in the OD_{600nm}) and every 10 seconds after stabilization for 30 seconds. Relative values were normalized to the effect of PBS, which was assigned a value of 0% and 100% isopropanol, which was assigned a value of 100%. All assays were performed a minimum of 3 times with representative data shown.

Erythrocyte hemolysis. Whole human blood was collected in EDTA and erythrocytes were separated by centrifugation. The cells were washed with 150 mM NaCl, and resuspended in PBS at a concentration of 10^8 erythrocytes/mL. The erythrocyte suspension was incubated at 37°C with increasing concentrations of $D(KLAKLAK)_2$. After centrifugation, the hemoglobin released in the supernate was measured at 415 nm after 48h. All assays were performed in triplicate with mean and standard error of the mean shown.

Surface charge in *E. faecalis* pair. Cell surface charge was measured using a modified cytochrome *c* assay as described previously (304). Briefly cells were grown overnight in brain-heart infusion broth (BHI), washed twice with 20 mM 3-(N-morpholino) propanesulfonic acid (MOPS) buffer pH 7, and resuspended in the same buffer. Cells were then incubated with 0.5 mg/ml cytochrome *c* for 10 minutes, pelleted and the amount of cytochrome *c* remaining in the supernatant was determined spectrophotometrically at 530 nm (the lower the amount of bound cytochrome *c*, the more positively charged the cell envelope). All assays were performed in triplicate with average and standard deviation shown.

CM permeabilization in *E. faecalis* pair. The highly sensitive LIVE/DEAD *BacLight* kit was used in order to measure DAP-mediated CM permeabilization. Cells were grown to mid-exponential phase in BHI, harvested and washed once in HEPES buffer (5mM HEPES, 5mM glucose pH 7.2), then resuspended in the same buffer containing 5 μ M SYTO-9, 30 μ M propidium iodide and 50 mg/L calcium. Cells were allowed to equilibrate for 10 min at room temperature and were added to 96-well plate with increasing concentrations of DAP. Fluorescence was measured immediately with an excitation wavelength of 488 nm and emission wavelength of 510 nm on a fluorescence spectrophotometer. A negative control of buffer and a positive control of 100% isopropanol were included. Percent fluorescence change was calculated setting isopropanol control as 100% fluorescence change and buffer as 0% fluorescence change. All assays were performed in triplicate with average and standard deviation shown.

CM depolarization in *E. faecalis* pair. We also monitored the effect of DAP on CM potential ($\Delta\Psi$) using diSC₃(5) using the protocols described above with the following modification: all assay buffers contained 50mg/L calcium. All assays were performed in triplicate with average and standard deviation shown.

Chapter III

$D(KLAKLAK)_2$: A Membrane Active Anti-Gram Negative Peptide

Chapter III

D(KLAKLAK)₂: A Membrane Active Anti-Gram Negative Peptide

Introduction

(KLAKLAK)₂ is a KLA-peptide that was originally assessed by the McLaughlin group for its ability to kill both eukaryotic and prokaryotic cells (289). Jadvapoor et al. designed several different amphipathic helical peptides with the sequences: (KLAKKLA)_n, (KLAKLAK)_n (where n = 1,2,3), (KALKALK)₃, (KLGKKLG)_n, and (KAAKKAA)_n (where n = 2,3) to determine the effect of length, sequence, and structure on biological activity. It was found that all of the leucine- and alanine-containing peptides of the same length had similar MIC values. In addition, it was shown that the 7-mers were inactive and the leucine-containing 21-mers were 2 times as potent at killing bacteria as the 14-mers, while maintaining low mammalian-cell toxicity (289). Peptide cytotoxicity against human erythrocytes and 3T3 mouse fibroblasts and antibacterial activity against *E. coli*, *P. aeruginosa*, and *S. aureus* was tested. All of the leucine- and alanine-containing peptides of the same length were found to have similar MIC values and it was also found that the peptides were much less lytic toward human erythrocytes than 3T3 cells. Since the first description, (KLAKLAK)₂ has been widely used in cancer research (305, 306, 307, 308, 309) as a pro-apoptotic moiety. Once internalized into eukaryotic cells, AMPs such as (KLAKLAK)₂ cause mitochondrial disruption by triggering membrane permeabilization and swelling, resulting in release of cytochrome *c* and induction of apoptosis (306).

Here, we show that (KLAKLAK)₂, is an effective antimicrobial against Gram-negative laboratory strains and clinical isolates. Despite the therapeutic promise, AMPs development is hindered by their susceptibility to proteolysis. Here, we demonstrated that an all-D enantiomer of (KLAKLAK)₂, resistant to proteolysis, retains its activity against Gram-negative pathogens. In addition, we have elucidated the specific site and mechanism of action of _D(KLAKLAK)₂ through a repertoire of whole-cell and model membrane assays.

Results

_D(KLAKLAK)₂ peptido-mimetic is proteolytically stable

We used mass spectrometry to determine the stability of _D(KLAKLAK)₂ compared to _L(KLAKLAK)₂ in the presence of human liver microsome extracts containing Cytochrome P450 (CYP450), the major enzyme complex involved in drug metabolism. To control for specificity, reactions were carried out in the presence or absence of the NADPH co-factor. (KLAKLAK)₂ peptide degradation in the absence of NADPH would indicate metabolism from enzymes other than CYP450. Intact _D(KLAKLAK)₂ was detected after 0, 30, 60 minutes and 24 hours confirming its prolonged stability after continuous exposure to proteases (Fig. 3-1). All samples co-incubated with NADPH contained a small amount of an unidentified adduct at +258 amu. A significant amount of an unidentified adduct at +105 amu was found after 24 hours specifically in the samples containing NADPH. (Fig. 3-2 and 3-3).

Contrastingly, the concentration of _L(KLAKLAK)₂ decreased over time and it was completely degraded after 24 hours of exposure to liver microsome fractions,

regardless of NADPH presence. Both N-terminal and C-terminal degraded products of $_L(KLAKLAK)_2$ were found at 10 min, 30 min, and 60 min, and their identity was verified by MS-MS profile. At 24 hours, some degraded products were still detected in the samples with microsomes, but without NADPH. However, in the presence of NADPH, degradation products were absent. The fact that there was no significant difference between samples with and without NADPH, indicates that the degradation is likely the result from metabolism from enzymes other than CYP450.

$_D(KLAKLAK)_2$ peptido-mimetic exhibits Gram-negative bactericidal activity

We determined the spectrum of $_D(KLAKLAK)_2$ activity against a variety of bacteria and mold species using the broth dilution method published by the Clinical and Laboratory Standards Institute (CLSI) in the USA (CLSI document M100-S16CLSI), and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) in Europe (310). Table 3-1 summarizes the $_D(KLAKLAK)_2$ minimum inhibitory concentration (MIC) measured for each strain tested. These data suggest that $_D(KLAKLAK)_2$ is effective against Gram-negative bacteria such as *P. aeruginosa*, *A. baumannii*, *K. pneumoniae* and *E. coli*. Molds such as *Rhizopus oryzae* and *Mucor sp.* and yeasts such as *Candida sp.* and the fungus *Cryptococcus albidus* were also found to be sensitive to treatment with $_D(KLAKLAK)_2$. However, in this study, we investigated only $_D(KLAKLAK)_2$ activity against Gram-negative bacteria.

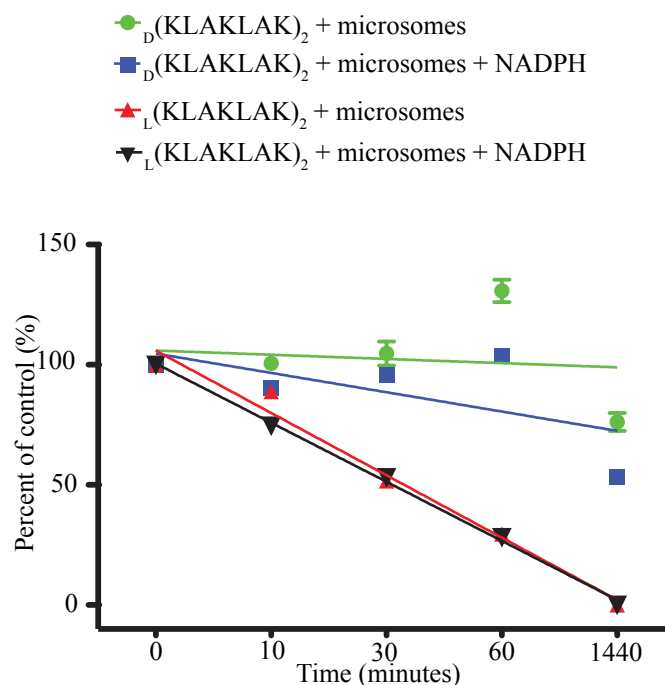


Figure 3-1. $D(KLAKLAK)_2$ is resistant to degradation by human liver microsomes. Intact $D(KLAKLAK)_2$ was detected 0, 10, 30, 60 minutes and 24 hours after continuous exposure to microsomal enzymes in the presence (blue line) and absence of NADPH (green line). $L(KLAKLAK)_2$ concentrations decreased rapidly over time with complete degradation observed within 24 hours of exposure to liver microsome fractions both in the presence (black line) and absence (red line) of NADPH.

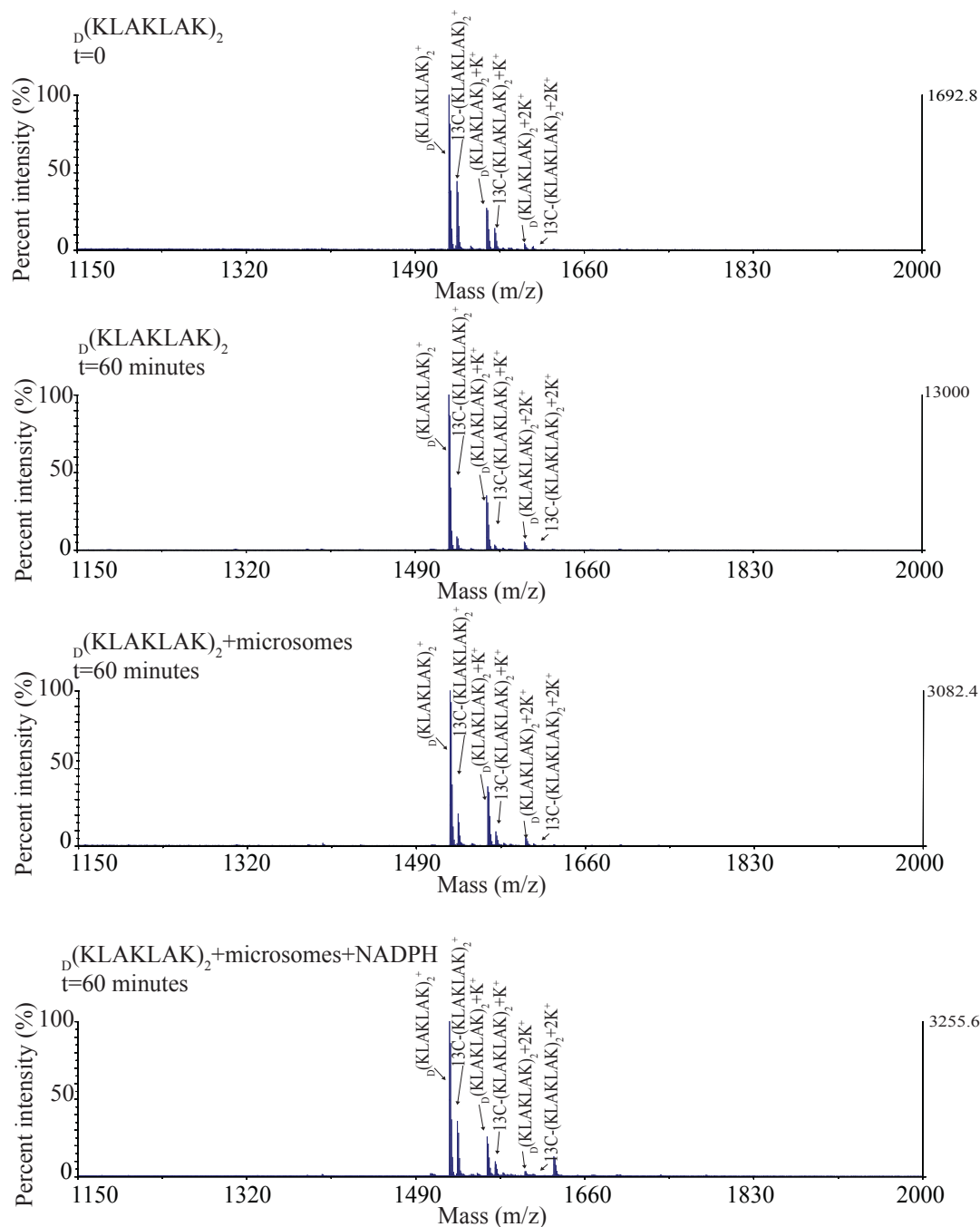


Figure 3-2. $D\text{-(KLAKLAK)}_2$ is resistant to degradation by human liver microsomes. No degradation products of $D\text{-(KLAKLAK)}_2$ were found for up to 24 hours post continuous exposure to microsomal enzymes. Representative mass spectra are shown at (A) pre-incubation; (B) 60 minutes in buffer at 37°C; (C) 60 minutes post incubation with pooled human liver microsomes and (D) 60 minutes post incubation with pooled human liver microsomes in the presence of NADPH.

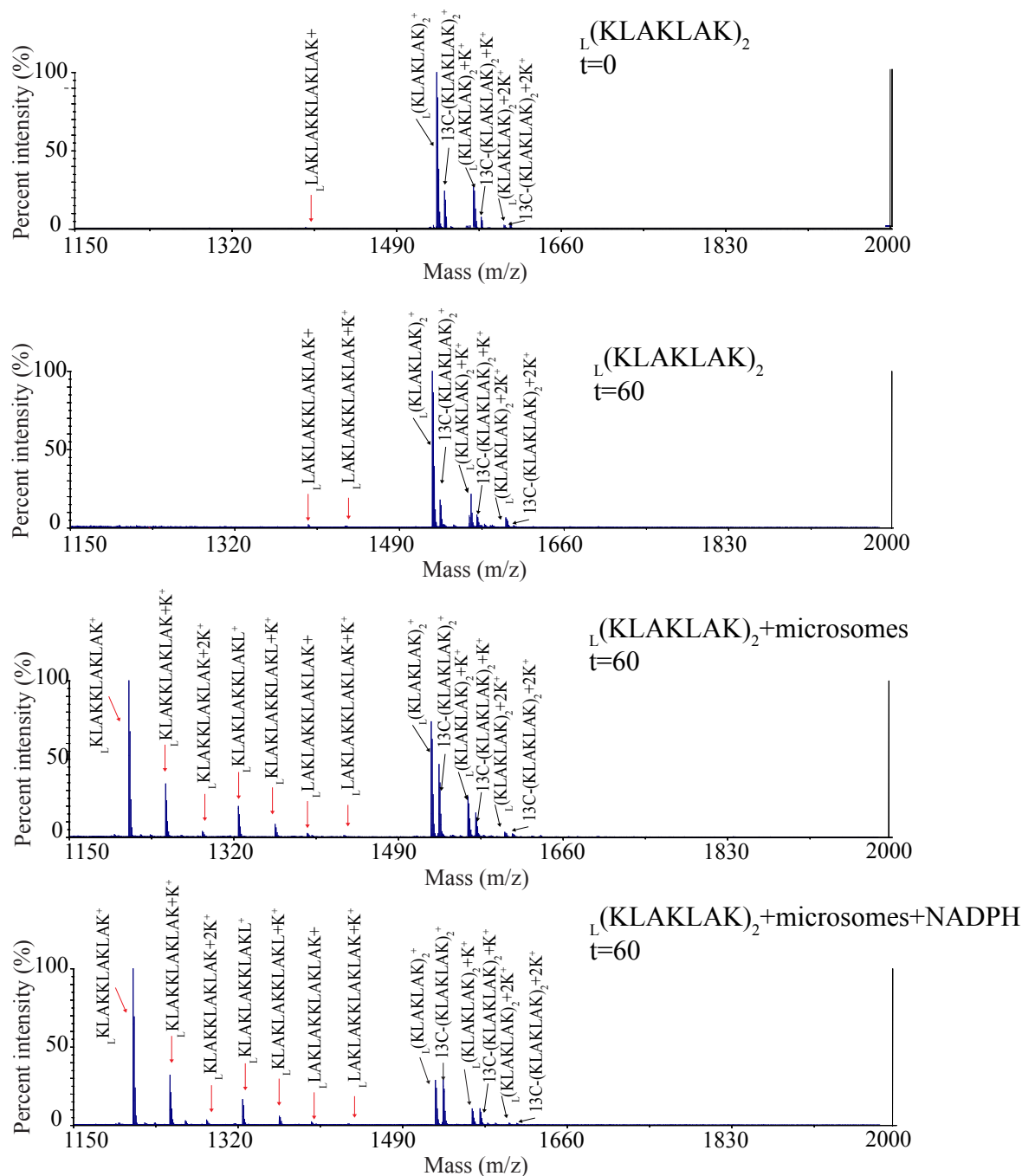


Figure 3-3. $L(KLAKLAK)_2$ is rapidly degraded during exposure to human liver microsomes. Degradation products resulting from both N-terminal and C-terminal proteolysis of $L(KLAKLAK)_2$ were found at 10 min, 30 min, and 60 min. Representative mass spectra are shown at (A) pre-incubation; (B) 60 minutes in buffer at 37°C; (C) 60 minutes post incubation with pooled human liver microsomes and (D) 60 minutes post incubation with pooled human liver microsomes in the presence of NADPH.

Table 1. MIC values for selected organisms tested for $D(KLAKLAK)_2$ susceptibility.

Organism	MIC $\mu\text{g/ml}$ $D(KLAKLAK)_2$
<i>Acinetobacter baumannii</i> ATCC 19606	300
<i>Pseudomonas aeruginosa</i> (PAO1)	75
<i>Pseudomonas aeruginosa</i> (PA14)	75
<i>Escherichia coli</i> ATCC 25922	150
<i>Klebsiella pneumoniae</i>	300
<i>Stenotrophomonas maltophilia</i>	300
<i>Cryptococcus albidus</i>	150
<i>Candida albicans</i>	300
<i>Staphylococcus aureus</i>	Not effective
<i>Staphylococcus epidermidis</i>	Not effective
<i>Mucor</i> sp.	300
<i>Rhizopus oryzae</i>	300
<i>Aspergillus fumigatus</i>	Not effective
<i>Fusarium oxysporum</i>	Not effective
<i>Fusarium solani</i>	Not effective
<i>Scedosporium prolificans</i>	Not effective
<i>Aspergillus terreus</i>	Not effective

Gram-negative bacterial growth is inhibited within 4 hours after exposure to $D(KLAKLAK)_2$

Although widely used to determine antibiotic efficacy, MIC measurements do not provide information about the dynamic interaction between the drug and bacteria. To begin to understand the kinetics of this interaction, we compared time-kill curves of four different strains at multiples of MIC for a period of 24 hours. $D(KLAKLAK)_2$ treatment induced significant bacterial growth inhibition ranging between two to four logs, beginning at 4 hours after exposure. The effect of $D(KLAKLAK)_2$ peptido-mimetic was specific and dose- and time-dependent. However, substantially distinct killing rates were observed among species (Fig. 3-4).

Considering the potential therapeutic use of this peptide, we next examined whether $D(KLAKLAK)_2$ exerts bactericidal activity against clinical isolates and if its activity correlates with already acquired antibiotic resistance. We determined the MIC of 89 strains of *P. aeruginosa*, *K. pneumoniae*, and *E. coli*, respectively. Susceptibility profiles indicated that the average MIC extends from 150 $\mu\text{g/ml}$ for *E. coli* and *P. aeruginosa* to 600 $\mu\text{g/ml}$ for *K. pneumoniae*, respectively (Fig. 3-5). Comparative analysis of routinely tested antibiotics susceptibility profiles (Table 3-2) and $D(KLAKLAK)_2$ effect revealed no correlation between MDR and peptide susceptibility. For instance, five *E. coli* clinical isolates are sensitive to all antibiotics tested, yet they display average sensitivity to $D(KLAKLAK)_2$ (150 $\mu\text{g/ml}$). Similarly, one *E. coli* clinical isolate found to be resistant to most antibiotics tested, also exhibits average sensitivity to $D(KLAKLAK)_2$ (150 $\mu\text{g/ml}$). Among the twenty-five *P. aeruginosa* strains tested, four were resistant to 5 to 8 antibiotics. However, these strains exhibited different

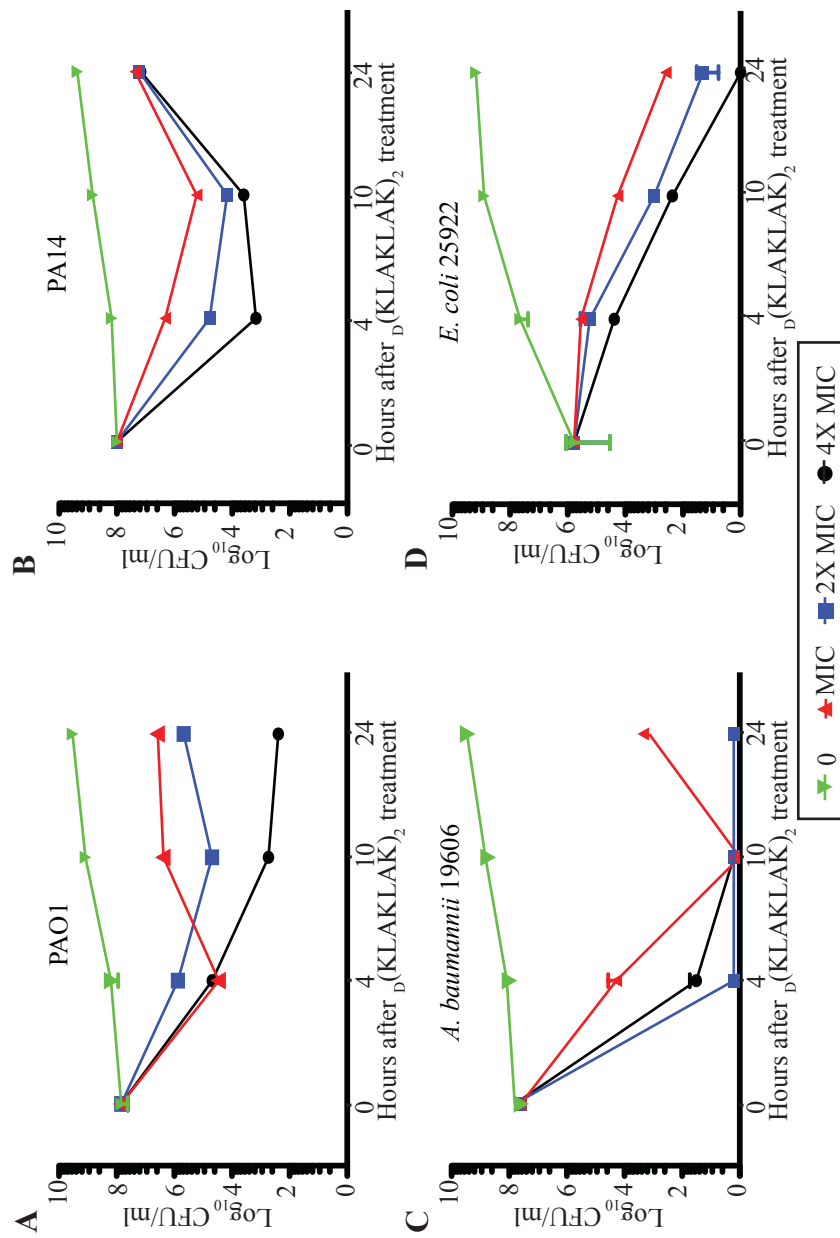


Figure 3-4. $(\text{KLAKLAK})_2$ inhibits growth of bacterial isolates within 4 hours of exposure. Survival curves of *PAOI*, *PA14*, *A. baumannii* 19606 and *E. coli* 25922 after $(\text{KLAKLAK})_2$ treatment at MIC (red line) 2X MIC (blue line) and 4X MIC (black line). $(\text{KLAKLAK})_2$ induced bacterial growth inhibition within 4 hours of treatment when compared to the untreated sample (green line).

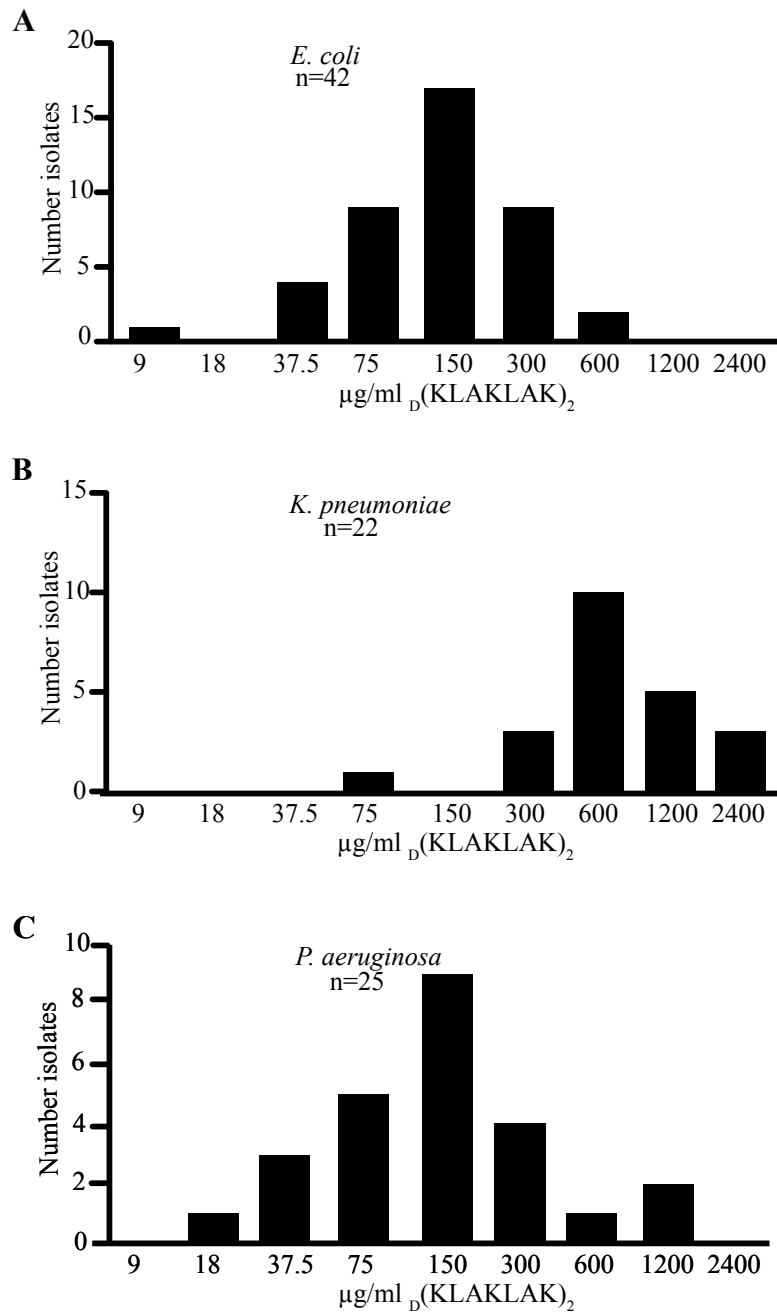


Figure 3-5. $_D(KLAKLAK)_2$ exerts bactericidal activity against clinical isolates. MIC of clinical strains of *E. coli* (A), *K. pneumoniae* (B), and *P. aeruginosa* (C) represented in µg/ml.

Table 2. Clinical isolates resistance profiles. Red highlight indicates isolates specifically mentioned in the text.

	Number of <i>E. coli</i> Isolates Resistant to Routinely Tested Antibiotics				
$D(KLAKLAK)_2$ MIC $\mu\text{g/ml}$	0	1-4	5-8	9-12	13-16
37	0	3	0	0	0
150	5	9	2	1	0
300	3	4	1	0	0
	Number of <i>P. aeruginosa</i> Isolates Resistant to Routinely Tested Antibiotics				
$D(KLAKLAK)_2$ MIC $\mu\text{g/ml}$	0	1-4	5-8	9-11	13-14
37	2	0	0	1	0
150	2	4	3	0	0
1200	1	0	1	0	0
	Number of <i>K. pneumoniae</i> Isolates Resistant to Routinely Tested Antibiotics				
$D(KLAKLAK)_2$ MIC $\mu\text{g/ml}$	0	1-4	5-8	9-12	13-14
75	0	0	0	0	1
600	0	10	0	0	0
1200	0	5	0	0	0

susceptibilities to D(KLAKLAK)_2 , spanning from the lowest to the second highest concentration tested. The most striking example is represented by a *K. pneumoniae* clinical isolate found to be resistant to all antibiotics tested, yet highly sensitive to D(KLAKLAK)_2 (75 $\mu\text{g/ml}$).

Combination therapy has been shown to enhance antibiotic effectiveness and to contribute less frequently than monotherapy to selection of resistance. We hypothesized that D(KLAKLAK)_2 may have synergistic activity with different classes of antibiotics. As an example, we performed an *in vitro* time-kill assays to determine the activity of D(KLAKLAK)_2 in combination with piperacillin. Our results indicated a significant synergistic effect within 4 hours after treatment at one half time the MIC for both peptide and the conventional antibiotic piperacillin (Fig. 3-6).

D(KLAKLAK)_2 activity is growth type and stage independent

Antibiotic susceptibility is routinely tested on planktonic microorganisms during exponential growth phase. However, certain types of infections are caused by bacteria growing as a biofilm. Adherent growth renders microorganisms 100 to 1000 times less affected by antibiotics. To determine whether D(KLAKLAK)_2 is active against bacterial biofilms, we exposed a 24 hour old *P. aeruginosa* biofilm to increasing concentrations of peptide. Similar to regular use antibiotics, D(KLAKLAK)_2 was less effective against *P. aeruginosa* biofilm (400 $\mu\text{g/ml}$) compared to free-growing cells (150 $\mu\text{g/ml}$). Notably, the increase in susceptibility is less than 3 times suggesting that the peptide diffuses efficiently through the biofilm and it is active against the metabolically active cells present at the outer surface of the biofilm as well as the slow growing bacteria present in

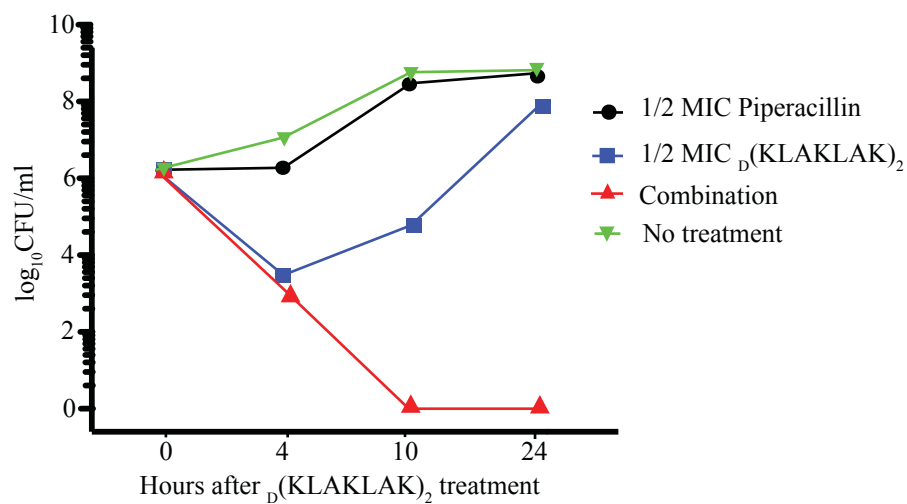


Figure 3-6. Synergistic effect of $D(KLAKLAK)_2$ and piperacillin. Time-killing curve showed that a combination of 1/2 MIC of $D(KLAKLAK)_2$ with 1/2 MIC piperacillin (red line) significantly enhanced the bactericidal effect when compared to the activity of each drug alone: piperacillin, black line and $D(KLAKLAK)_2$ blue line.

the inner layers. Moreover, the activity of $_D(KLAKLAK)_2$ was found to be similar in cation-adjusted Muller-Hinton and 10% fetal bovine serum RPMI suggesting that the effects of $_D(KLAKLAK)_2$ are not media dependant (Fig. 3-7).

We subsequently reasoned that $_D(KLAKLAK)_2$ might also be active against stationary growth phase bacteria. The ability of peptide to kill bacteria was measured using the LIVE/DEAD *BacLight* kit. This assay employs a combination of two nucleic acid stains used to distinguish live bacteria with an intact membrane from dead bacteria. Live cells stained green with SYTO9 while dead bacteria stained red and yellow due to SYTO9 and propidium iodide.

Unlike most conventional antibiotics that are active only against metabolically active cells, the peptide equally affected exponential and stationary growth phase *P. aeruginosa* (Fig. 3-8).

$_D(KLAKLAK)_2$ causes morphological membrane damage

In considering the amphipathic peptide mode of action, we first hypothesized that the peptide might induce gross bacterial membrane damage. Electron micrographs of *P. aeruginosa* incubated with freshly prepared $_D(KLAKLAK)_2$ at increasing concentrations revealed clear morphologic evidence of membrane damage (Fig. 3-9).

To elucidate the underlying mechanism of action, we employed the lysozyme lysis assay to determine outer membrane loss of integrity. When the bacterial membrane is damaged, lysozyme gains faster access to its peptidoglycan substrate inducing rapid cell lysis. *A. baumannii* 19606 and *P. aeruginosa* were exposed to increasing concentrations of $_D(KLAKLAK)_2$ in the presence of lysozyme. The synthetic peptide

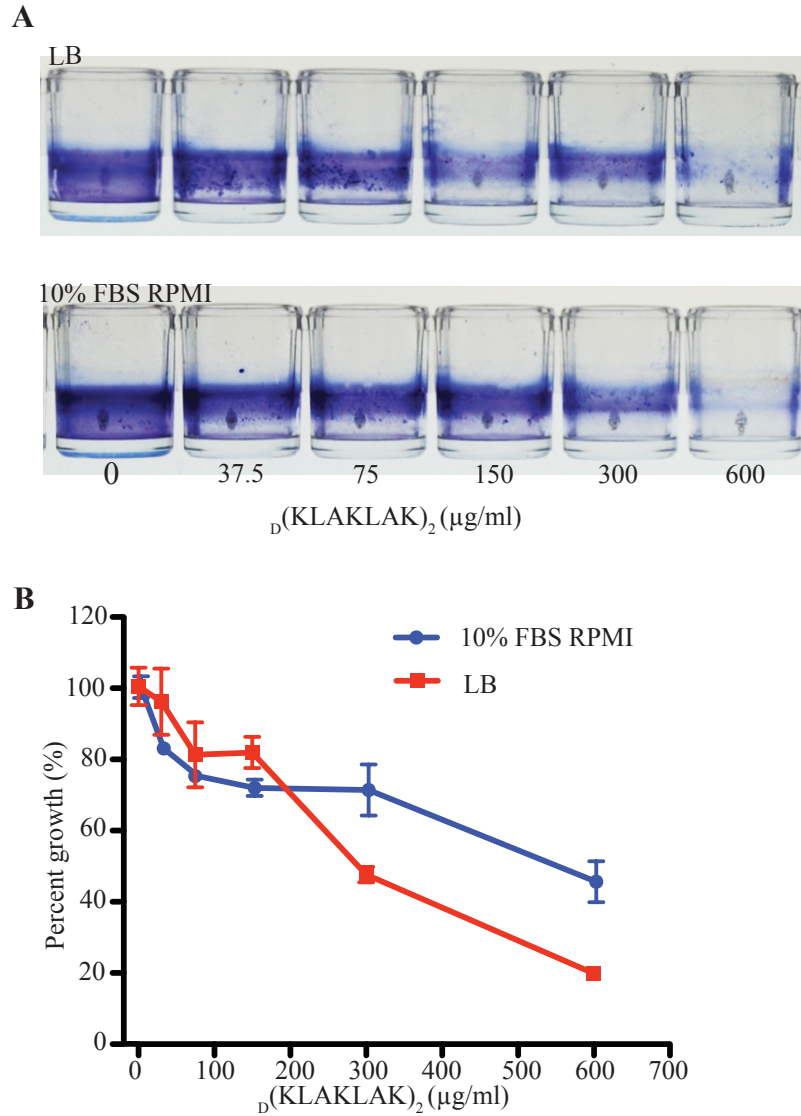


Figure 3-7. $_D(\text{KLAKLAK})_2$ inhibits biofilm growth. Twenty-four hour-old biofilm grown in LB (A) or 10% FBS RPMI (B) was treated with increasing concentrations of $_D(\text{KLAKLAK})_2$. Both visual (A,B) and quantitative (C) assessment demonstrated dose-dependent eradication of biofilm.

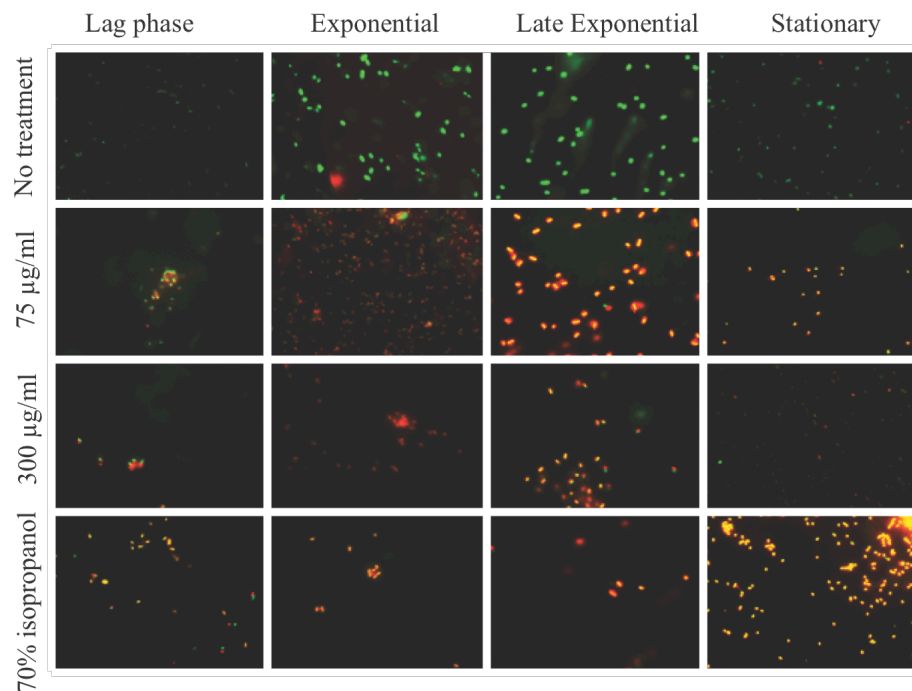


Figure 3-8. $_D(KLAKLAK)_2$ activity is growth stage independent.
 The ability of $_D(KLAKLAK)_2$ to cause membrane permeability in PA14 was measured in lag, exponential, late exponential and stationary phase. Clear evidence of membrane damage is represented by yellow or red nucleic acid staining when compared to the green nucleic acid staining (no treatment control).

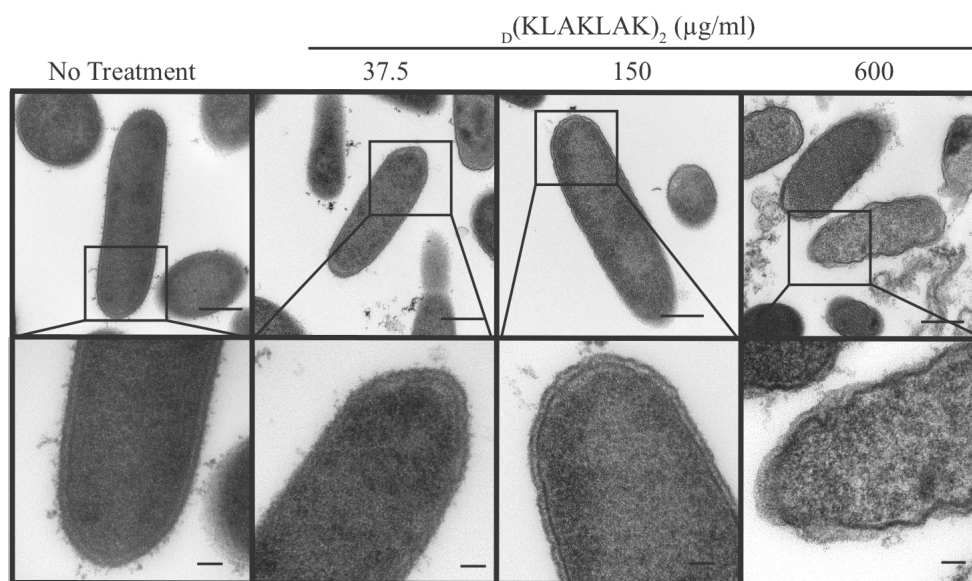


Figure 3-9. $_D(KLAKLAK)_2$ induces morphological membrane damage. TEM images of *P. aeruginosa* after 25 minutes of $_D(KLAKLAK)_2$ treatment showed clear morphological membrane damage starting after treatment with 37.5 $\mu\text{g/ml}$ peptide.

facilitated rapid lysozyme-induced cell lysis in a concentration dependant manner. *P. aeruginosa* was completely lysed when exposed to 16 µg/ml of peptide while only 60% of the *A. baumannii* cells were destroyed at the same concentration. In contrast, a control peptide had no effect on the bacterial membrane (Fig. 3-10). Since the MICs for *A. baumannii* 19606 and *P. aeruginosa* are similar, genus-specificity suggests the possibility that the peptide activity is time dependent.

Severe membrane injury usually results in membrane potential dissipation and cell death due to loss of membrane function. We next sought to assess whether the loss of membrane integrity results in membrane depolarization using the membrane potential-sensitive dye diSC₃(5). Bacterial exposure to D(KLAKLAK)₂ induced a dose dependant loss of membrane potential in all strains tested. However, a control peptide had no effect on the bacterial membrane suggesting that D(KLAKLAK)₂ activity is specific (Fig. 3-11).

D(KLAKLAK)₂ exerts its effect on membrane lipids

We next sought physical evidence of interaction between the peptide and cell membrane lipids. Giant unilamellar vesicles derived from *E. coli* extract devoid of membrane proteins were treated with 3 µg of D(KLAKLAK)₂. Upon exposure to the peptide, the liposomal membrane appeared punctuated suggesting lipid dissolution perhaps as a result of D(KLAKLAK)₂ insertion into the lipid bilayer (Fig. 3-12). This effect was not seen with a control peptide (data not shown).

To further demonstrate lipid membrane dissolution, we investigated the ability of D(KLAKLAK)₂ to disrupt dual labeled liposomes using fluorescence resonance energy

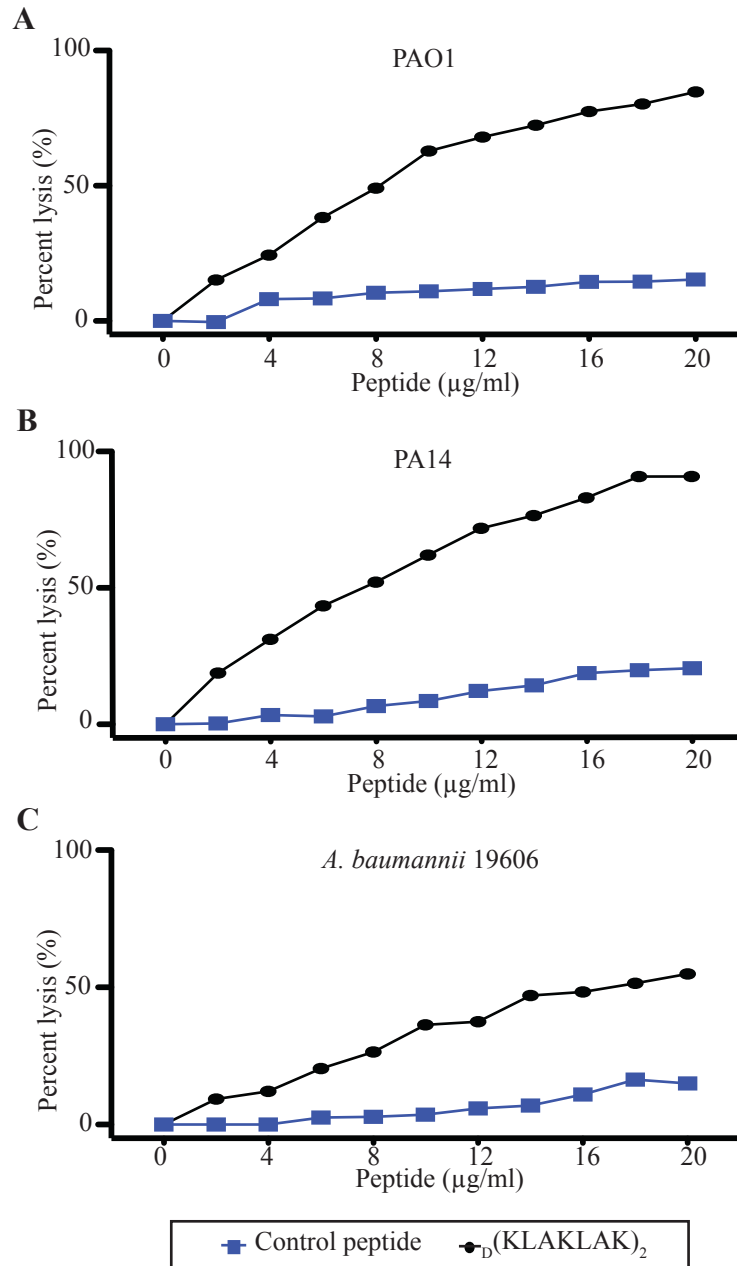


Figure 3-10. $_D(KLAKLAK)_2$ causes dose dependant membrane permeability. $_D(KLAKLAK)_2$ induces membrane permeability in PAO1, PA14, and *A. baumannii* 19606 (A, B, C black lines, respectively). A control peptide (blue line) did not affect the membrane.

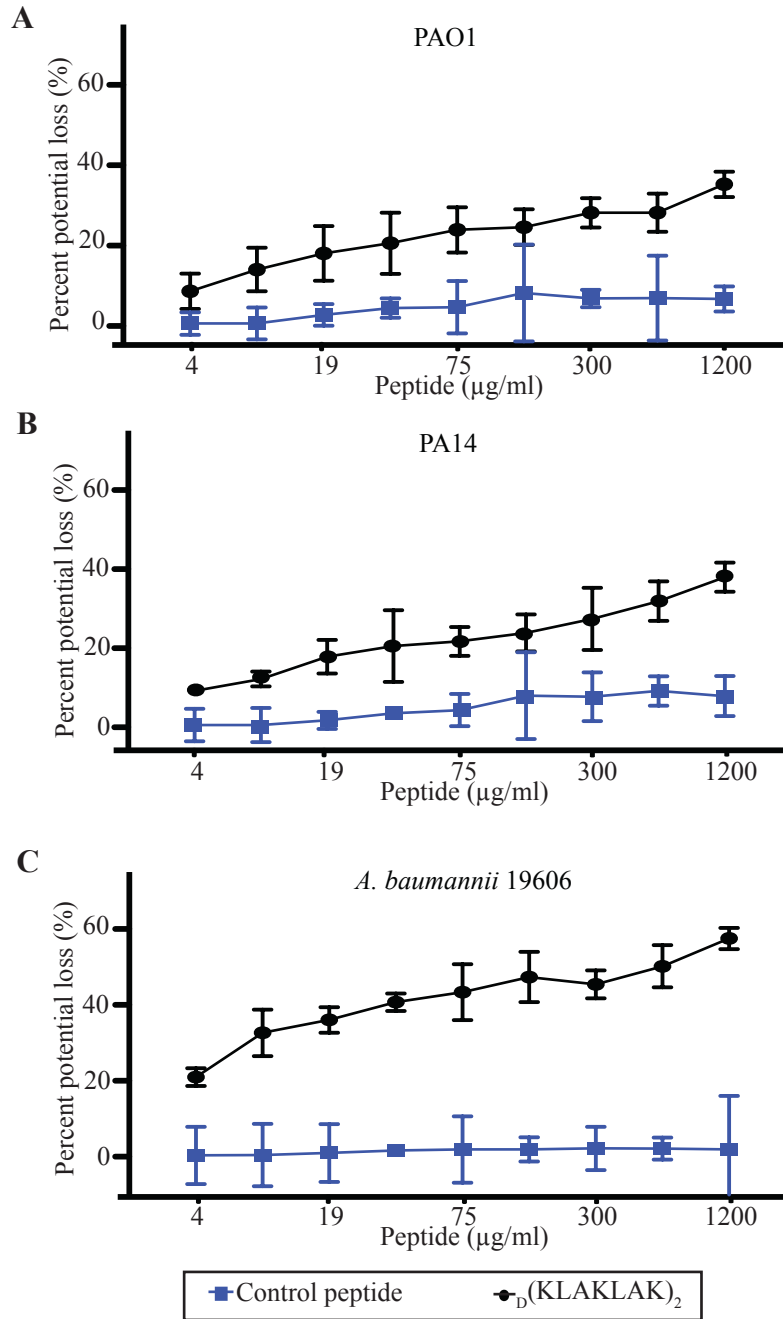


Figure 3-11. $_D(KLAKLAK)_2$ treatment induces dose dependent loss of membrane potential. $_D(KLAKLAK)_2$ causes loss of membrane potential in PAO1, PA14, and *A. baumannii* 19606 (A, B, C black lines, respectively) when compared with a control peptide (blue line).

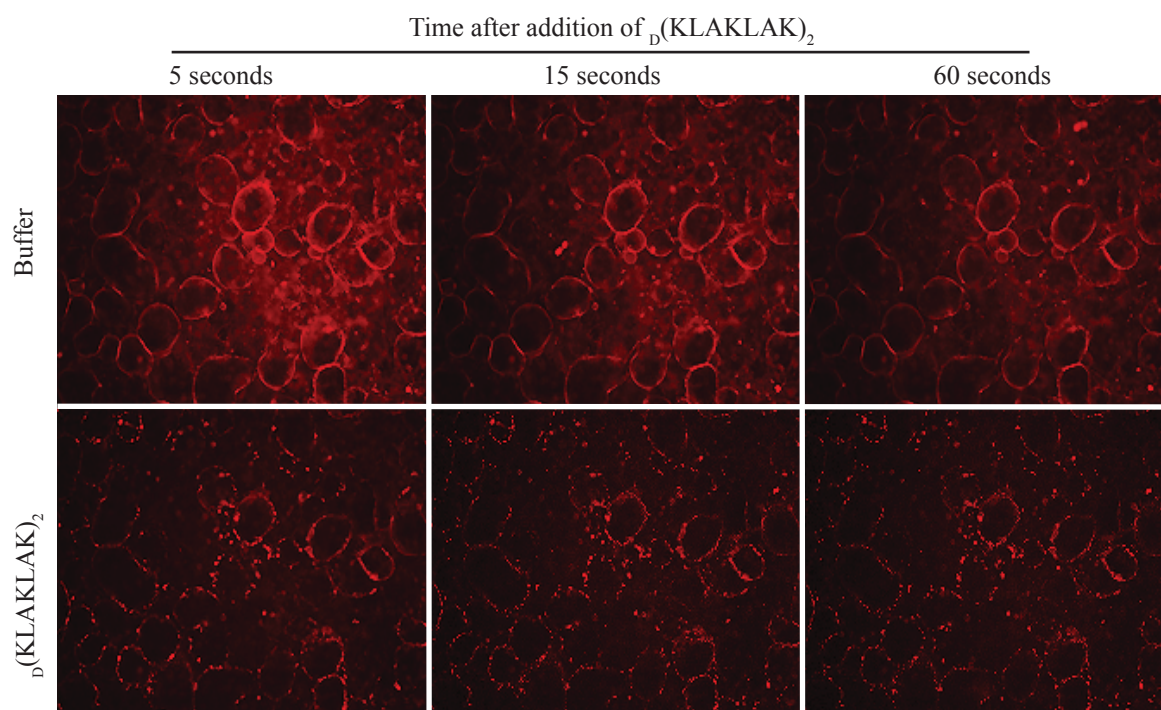


Figure 3-12. $_D(KLAKLAK)_2$ exerts its effect on lipid bilayers devoid of membrane proteins. Giant unilamellar vesicles derived from *E. coli* extract devoid of membrane proteins treated with 3 μ g of $_D(KLAKLAK)_2$ (bottom row) showed clear punctate staining, evidence of lipid membrane dissolution when compared to vehicle alone (top row).

transfer (FRET). *E. coli* extract-derived liposomes labeled with either Nitro-2-1,3-BenzoxaDiazol-4-yl (NBD) phosphatidylethanolamine (PE) (excitation at 460 nm and emission at 534 nm), or rhodamine PE (excitation at 550 nm and emission at 590 nm), were incubated with increasing concentrations of $_D(KLAKLAK)_2$. When the liposomes are intact, the two fluorescent lipids are not in close enough proximity to yield an efficient energy transfer. However, when $_D(KLAKLAK)_2$ disrupts the liposomal membrane, free lipids are able to come in close contact leading to FRET. Energy transfer causes a decrease in emission intensity at 534 nm (NBD emission) and a corresponding increase in the emission intensity at 590 nm (rhodamine emission). In support of our hypothesis, $_D(KLAKLAK)_2$ disrupted the integrity of liposomes indicated by the emergence of an emission peak at 590 nm with the corresponding reduction of the peak at 534 nm. A control peptide had no influence on liposome integrity whereas ethanol disrupted both type of liposomes resulting in an energy transfer similar with the one induced by $_D(KLAKLAK)_2$ (Fig. 3-13).

$_D(KLAKLAK)_2$ specifically disrupts bacterial membrane

Because the bacterial outer layer and eukaryotic membrane lipid composition are different, we next asked whether $_D(KLAKLAK)_2$ interacts only with lipids specific for the microbial outer layer. Phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and phosphatidylserine (PS) represent the most common bacterial lipids; whereas the eukaryotic cell membrane has a more sophisticated composition with phosphatidylcholine (PC) (24%) and cholesterol (17%) as major components. *E. coli* extract-derived labeled liposomes supplemented with increasing concentrations of each

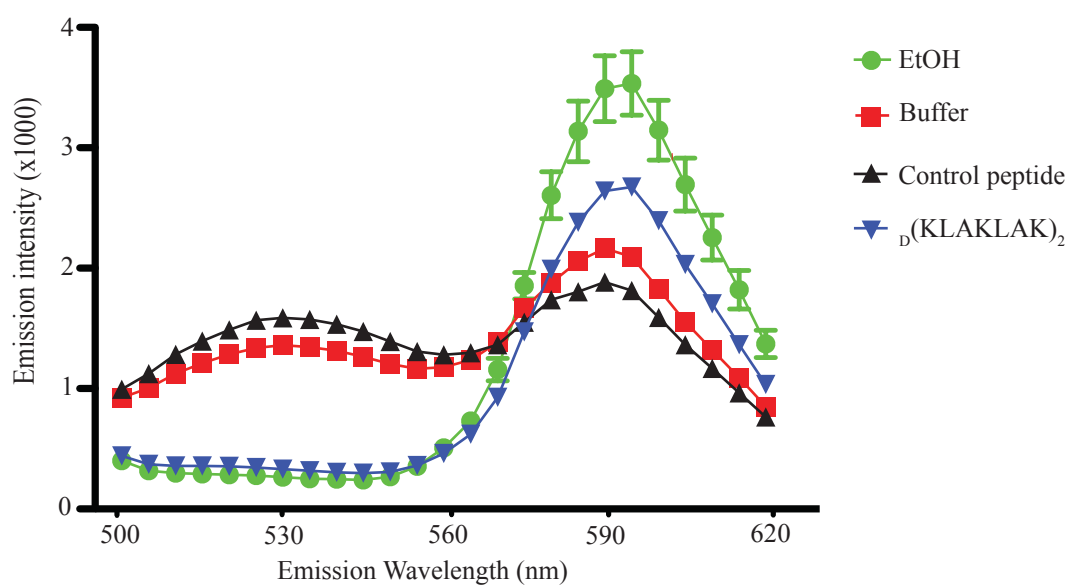


Figure 3-13. $D(KLAKLAK)_2$ disrupted the integrity of liposomes.
 $D(KLAKLAK)_2$ disrupted the integrity of labeled *E. coli* extract derived liposomes as indicated by the emergence of an emission peak at 590 nm with the corresponding reduction of the peak at 534 nm. A control peptide had no influence on liposome integrity whereas ethanol disrupted the liposomes resulting in an energy transfer similar with the one induced by $D(KLAKLAK)_2$.

specific lipids were incubated with $D(KLAKLAK)_2$ and assessed for FRET. Consistently, PE, PG and PS increased the susceptibility of liposomes to $D(KLAKLAK)_2$ in a dose dependent manner as demonstrated by a decrease in the peak at 534 nm complemented by an increase in the peak at 590 nm (Fig. 3-14, A, B, C). As expected, the addition of PC and cholesterol decreased the susceptibility of the *E. coli* extract liposomes to the peptide (Fig 3-14 D, Fig. 3-15).

To further demonstrate that $D(KLAKLAK)_2$ does not disrupt the eukaryotic membrane, we tested erythrocyte hemolysis after treatment with increasing concentrations of $D(KLAKLAK)_2$. Hemolysis was assessed at 1, 3, and 48 hours of exposure by measuring the absorbance hemoglobin release in the supernate at 415 nm. In support of our hypothesis, the peptide had no hemolytic activity even after 48 hours of treatment (Fig. 3-16).

Taken together, this data demonstrates that $D(KLAKLAK)_2$ causes outer membrane bilayer dissolution, which results in dissipation of membrane potential and bacterial death. Moreover, the peptide acts specifically on the bacterial membrane without disrupting the eukaryotic cell homeostasis.

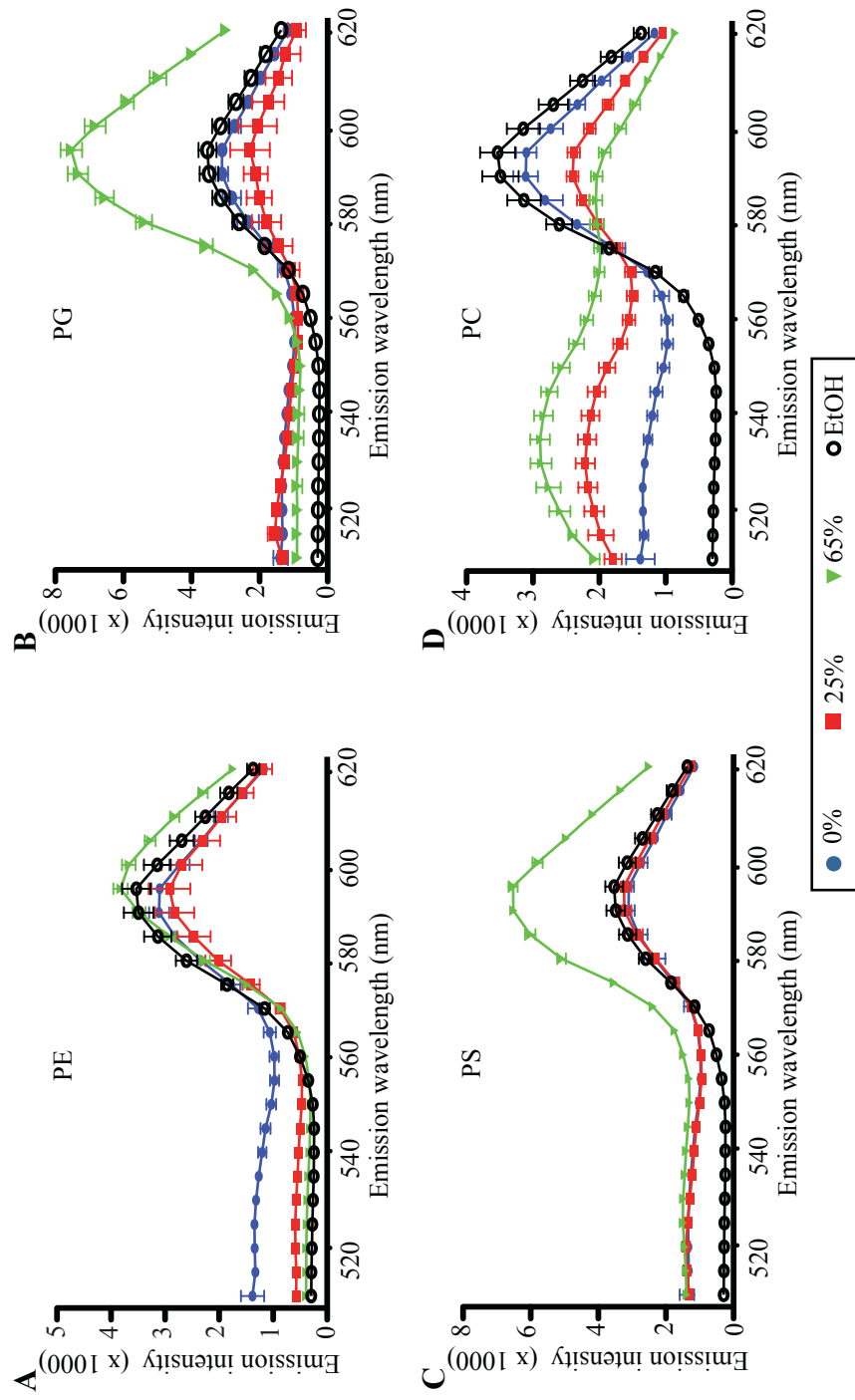


Figure 3-14. ${}_p(KLAKLAK)_2$ disrupted liposomes with composition similar to the bacterial membrane. ${}_p(KLAKLAK)_2$ disrupted the integrity of liposomes comprised of increasing percentages of PE, PG and PS (A, B, C, respectively) lipids specific for the microbial membrane. PC containing liposomes (D) were less affected by the peptide. Consistently, addition of PE, PG and PS (from 0-65%) increased the susceptibility of liposomes to ${}_p(KLAKLAK)_2$ in a dose dependent manner as demonstrated by a decrease in the peak at 590 nm complemented by an increase in the peak at 534 nm.

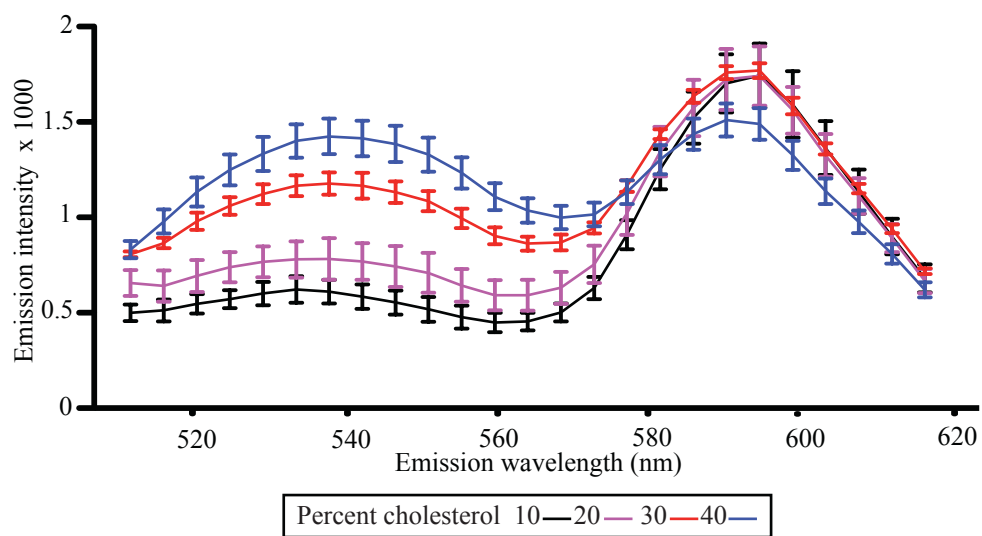


Figure 3-15. The effect of $D(KLAKLAK)_2$ on the lipid bilayer was significantly blunted with the addition of cholesterol to the liposomal membrane. Cholesterol decreased the susceptibility of the *E. coli* extract derived liposomes to the $D(KLAKLAK)_2$ treatment in a dose dependent manner as shown with a increase in the peak at 534 nm and a drop in the peak at 590 nm.

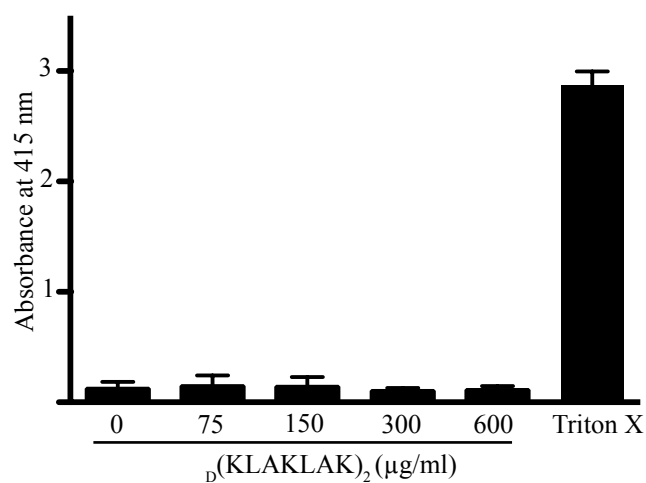


Figure 3-16. $D(KLAKLAK)_2$ does not cause erythrocyte hemolysis. Hemolysis of erythrocytes was measured via absorbance at 415 nm 48 hours after treatment with $D(KLAKLAK)_2$. The effect of $D(KLAKLAK)_2$ treatment was insignificant when compared to the vehicle (negative control) or Triton X-100 (positive control).

Chapter IV

Cell Envelope Analysis of Daptomycin-Susceptible *Enterococcus faecalis* and Its

Resistant Derivative that Arose During Therapy

Chapter IV

Cell Envelope Analysis of Daptomycin-Susceptible *Enterococcus faecalis* and Its Resistant Derivative that Arose During Therapy

Introduction

Enterococci are capable of causing a variety of potentially life-threatening infections and, in the USA, are ranked as the second most common cause of nosocomial infections after staphylococci (311). The current treatment of enterococcal infections has become an enormous challenge for clinicians due to the fact that this organism frequently exhibits multidrug resistance to the standard drugs of choice, namely ampicillin, aminoglycosides (high-level resistance) and vancomycin. Moreover, there has been a striking increase in the frequency of isolation and spread of VRE in hospitals around the world which significantly increase mortality, hospital stay and/or hospitalization costs (312). *Enterococcus faecium* is one of the “E’s” in the so-called ESKAPE pathogens, highlighted by the Infectious Diseases Society of America as problem pathogens requiring new therapies (313). Only two compounds are currently FDA-approved for the treatment of VRE infections, linezolid and quinupristin-dalfopristin. Both have important limitations for the treatment of severe VRE infections due to the frequent occurrence of clinical failures and recurrences, adverse toxicity profile, limited spectrum, bacteriostatic effect against VRE and increased reports of resistance. DAP is a lipopeptide antibiotic with *in vitro* bactericidal activity against enterococci which was approved in 2003 for the treatment of skin and soft tissue

infections (including for vancomycin-susceptible *E. faecalis*) and, in 2006, for the treatment of bacteremia and right-sided infective endocarditis caused by *Staphylococcus aureus*. Even though DAP does not have an indication for the treatment of VRE infections, it has *in vitro* bactericidal activity against enterococci (including ampicillin-resistant and VRE) and clinicians often use this compound off-label in severe enterococcal infections due to the lack of reliable options (314). A major drawback to the use of DAP for the treatment of VRE infections is the development of resistance during therapy (301, 315, 316). Although little is known about the mechanism of DAP resistance in enterococci, a critical step in DAP action is its interaction with the bacterial CM in a calcium-dependent manner.

Previous studies in *S. aureus* indicate that the development of DAP resistance is associated with important changes in the composition and function of the CM, cell envelope and, in some cases, CW (317). It has been shown that there are multifactoral pathways associated with DAP resistance in *S. aureus* and these pathways differ among resistant strains (317, 251, 318). In a recent study by Jones et al., using isogenic sets of *S. aureus* clinical isolates from a DAP-treated patient, a variety of CM and cell envelope alterations involving membrane fluidity, membrane phospholipid composition and asymmetry, and surface charge were found to be associated with the DAP-resistant phenotype (317). In addition to the phenotypic changes that have been described, there are many examples of alterations in the CW function and structure that have been proposed. In studies by Cui et al. (319) and Julian et al. (320) thickening in the CW was described suggestive of a physical barrier to DAP reaching the CM. Also, it has been

reported (321) that DAP-resistant *S. aureus* strains exhibit both (i) reduced membrane depolarization, and (ii) reduced daptomycin binding to the cytoplasmic membranes.

In addition to the phenotypic changes observed in DAP resistant *S. aureus* strains, genetic changes have also been identified. It has been found that mutations in the genes *mprF*, *yycG*, *rpoB* and *rpoC* are induced by *in vitro* passages in sub-inhibitory concentrations of DAP. These genetic mutations lead to changes in membrane charge through lysinylation of PG (322) alteration of membrane fatty acid biosynthesis, and changes in RNA polymerase, respectively.

Here we describe characterization of the CW and CM of a clinical pair of VRE *E. faecalis* isolates obtained from the blood of a patient with fatal bacteremia before (DAP-susceptible) and after (DAP-resistant) DAP therapy (DAP minimal inhibitory concentrations of 1 and 16 µg/ml, respectively). Our findings indicate that, similar to *S. aureus*, development of DAP-resistance in a vancomycin-resistant *E. faecalis* isolate is associated with alterations of the CW and properties of the CM. However, the genes linked to these changes in enterococci appear to be different from those described in *S. aureus*.

Results

Development of DAP resistance is associated with marked ultrastructural changes

TEM revealed important differences in the cell morphology of the two isolates. DAP-resistant cells tended to clump and formed aggregates with longer chains than DAP-susceptible cells. At higher magnifications, the presence of multiple septal

structures before complete cell separation was evident in the DAP-resistant cells (Fig. 4-1). The number of cells with a septum was consistently higher in the DAP-resistant isolate compared to the DAP-susceptible isolate ($P < 0.05$) (Fig. 4-2). Additionally, the CW thickness of the DAP-resistant isolate was greater than that of the DAP-susceptible isolate (average 18.12 ± 2.23 and $10.43 \text{ nm} \pm 1.34$, respectively, $p < 0.05$) (Fig. 4-3).

Alterations of CM potential are associated with the development of DAP-resistance

After determining that ultrastructural changes were present in the resistant *E. faecalis* isolate, we furthered our analysis of the cell membrane to determine if differences existed in DAP induced cell membrane potential loss. DAP dissipated the CM potential in a concentration-dependent manner after 5 min of exposure in DAP-susceptible cells. Conversely, this effect was significantly blunted in the DAP-resistant isolate (Fig. 4-4), indicating that emergence of DAP-resistance was associated with alterations in the ability to depolarize the target CM.

DAP-resistance is related to changes in DAP-induced permeability.

Using the LIVE/DEAD *BacLight* Kit, the DAP-susceptible isolate was shown to undergo a concentration-dependent increase in permeabilization after 5 min of DAP exposure. Conversely, the DAP-resistant isolate showed striking reductions of DAP-induced CM permeabilization (Fig. 4-5). This effect was seen at different time points from 10 min to 60 min.

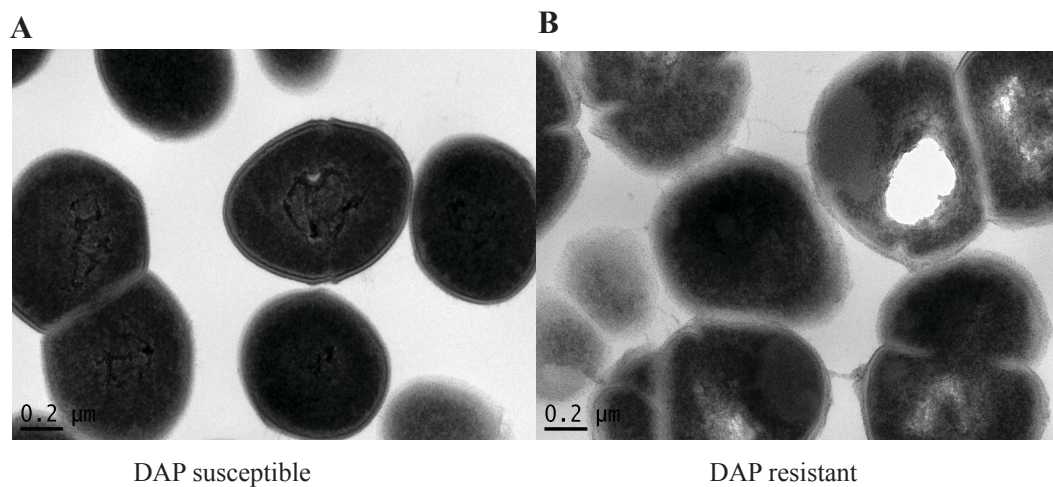


Figure 4-1. Ultrastructural changes of DAP-resistant isolate compared to DAP-susceptible parental strain. Electron micrographs revealed surface structures present on DAP-resistant (B) individual cells or connecting between cells not observed in the DAP-susceptible isolate (A).

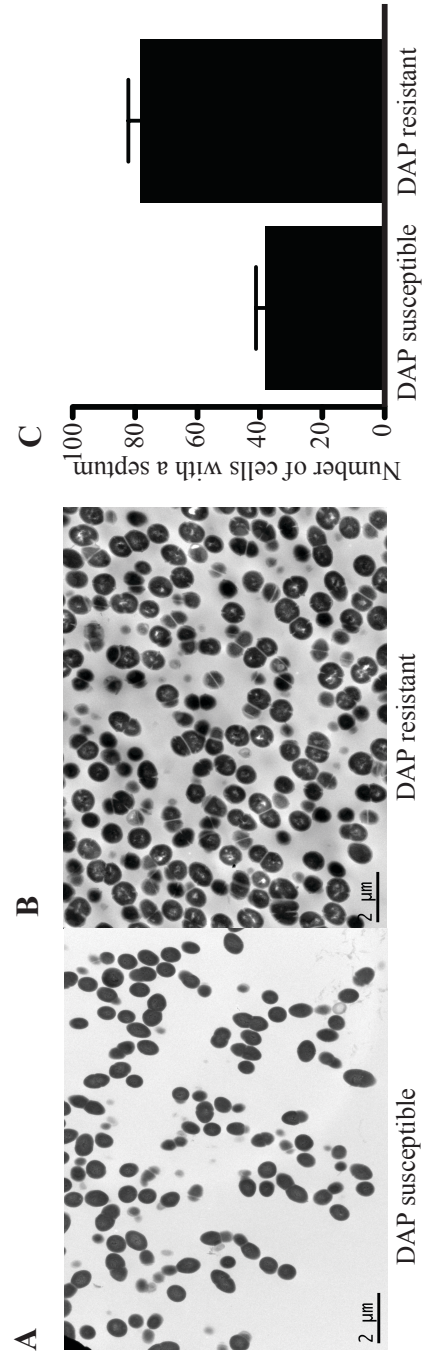


Figure 4-2. DAP-resistant isolate undergoes septal formation before complete separation. DAP-resistant isolates (B) appeared to be less able to separate after cellular division indicated by a higher percentage of cells with a septum (C) when compared to the DAP-susceptible parental strain (A).

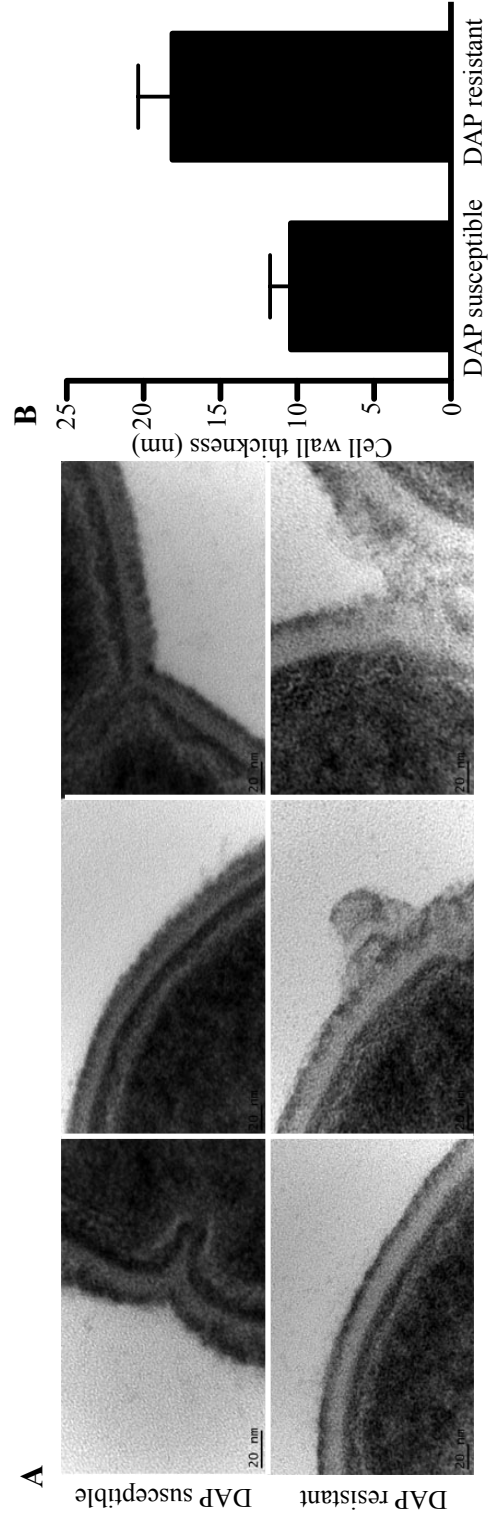


Figure 4-3. DAP-resistant isolate displays a thickened CW when compared to the DAP-susceptible parental strain. TEM micrographs of the DAP-resistant strain (A, bottom panel) reveals a thicker CW than the DAP-susceptible isolate (A, top row), average 18.12 \pm 2.23 and 10.43 nm \pm 1.34, respectively, $p < 0.05$.

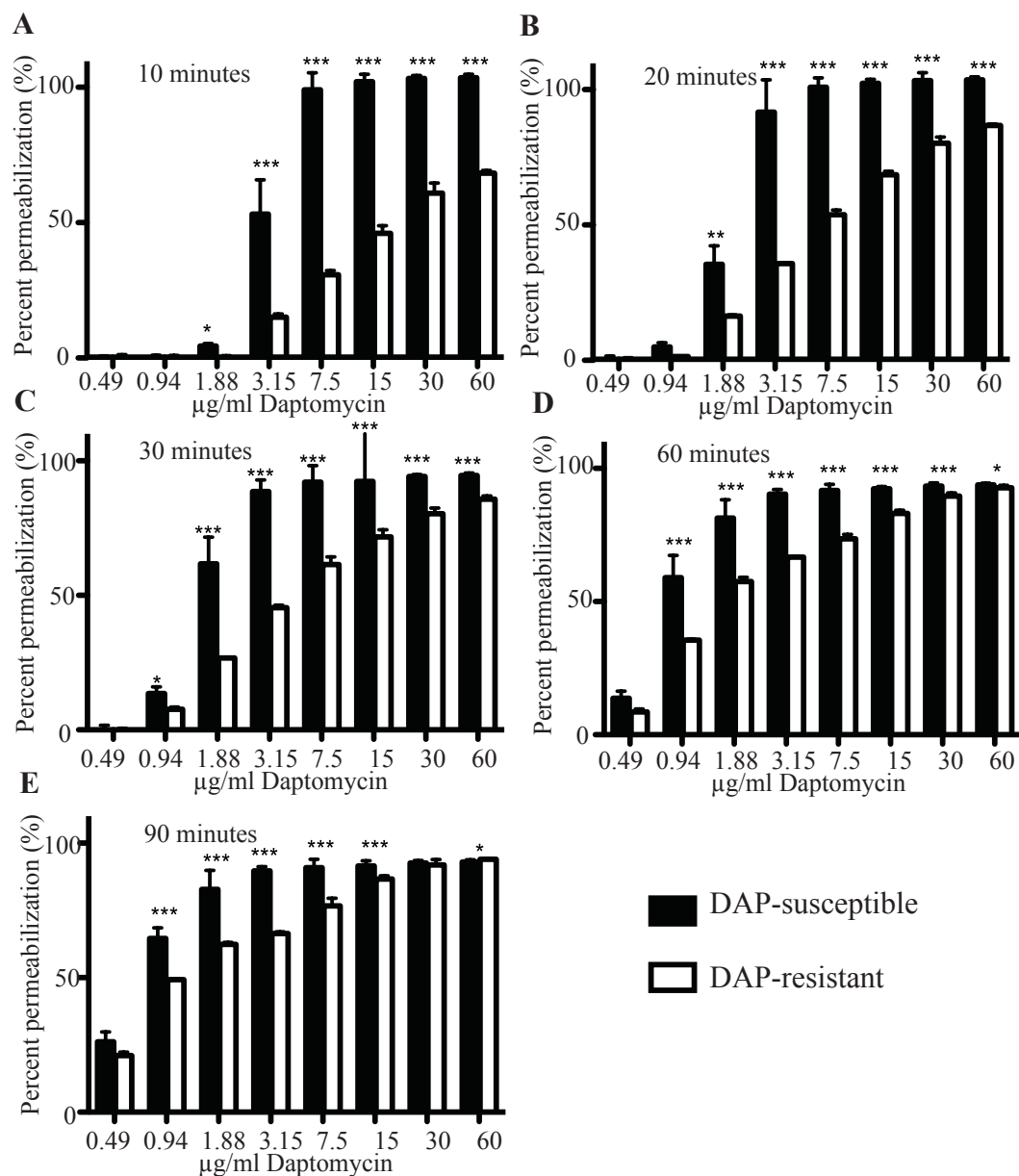


Figure 4-4. DAP-resistant isolate is less susceptible to DAP-induced membrane permeabilization. The DAP-resistant (open bars) isolate was less susceptible to DAP-induced membrane permeabilization when compared to the DAP-susceptible (filled bars) strain. The difference in susceptibility was seen as early as 10 minutes (A) after treatment with DAP at concentrations up to 60 µg/ml (5X the MIC of the DAP-resistant isolate). This trend continued at measurements made 20, 30, 60 and 90 minutes (B, C, D, E, respectively) after treatment.

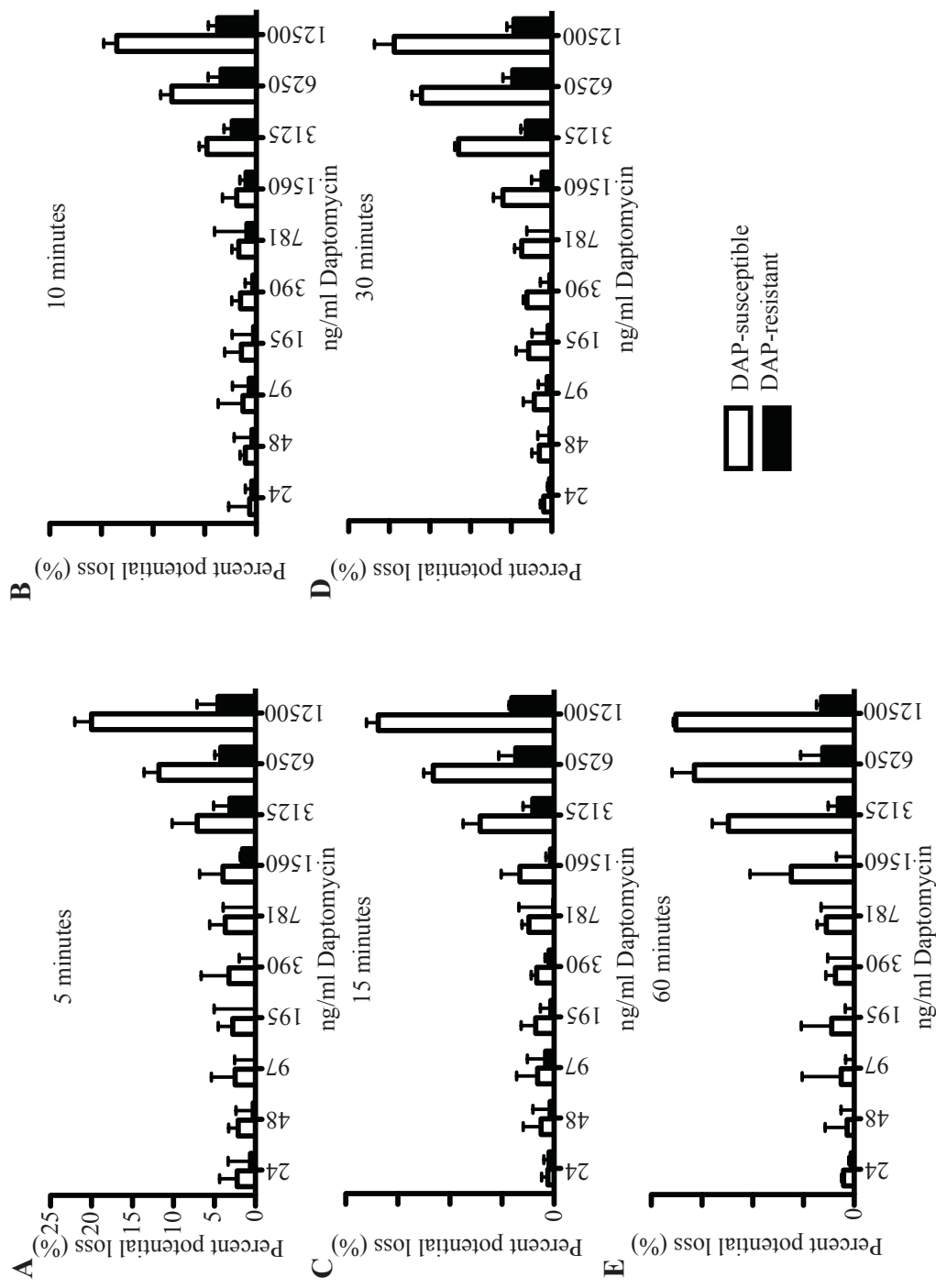


Figure 4-5. DAP-resistant isolate is less susceptible to DAP-induced membrane potential loss when compared to its DAP-susceptible parental strain. Induction of membrane potential loss measured via the DiSC3(5) assay due to treatment with DAP caused a rapid depolarization of the DAP-susceptible strain beginning 5 minutes after treatment (A, open bars). This effect was significantly reduced in the DAP-resistant (filled bars) at all time points tested (A, B, C, D, E).

Surface charge differences between DAP-resistant and DAP-sensitive *E. faecialis* isolates.

The overall surface charge in the DAP-resistant and DAP-sensitive isolates was investigated using the cationic cytochrome *c* molecule. Increased cell surface charge in DAP-resistance in *S. aureus* is well documented, and the surface charge appears to play an important role in the interaction of the antibiotic with the CM. It has been postulated that a more positively charged cell envelope can presumably “repel” cationic AMPs including DAP away from the CM and contribute to the development of resistance (317). DAP-resistant cells had significantly higher amount of unbound cytochrome *c*, indicating a more positively charged surface vs. the DAP-susceptible cells ($P < 0.05$) (Fig 4-6).

Collectively, our results suggest that development of DAP-resistance *in vivo* in *E. faecalis* is associated with profound ultrastructural changes in the CM, septal apparatus and CW surface charge, DAP-induced membrane permeabilization and potential loss. Taken together, all of these alterations likely enhance the organism’s ability to resist CM damage caused by the positively charged calcium-associated DAP.

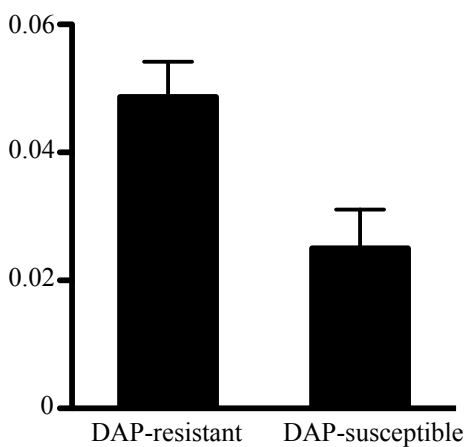


Figure 4-6. DAP-resistant cell surface more positively charged. DAP-resistant cells had significantly higher amount of unbound cytochrome *c*, indicating a more positively charged surface in comparison to the DAP-susceptible cells ($p < 0.05$).

Chapter V

Discussion

Chapter V

Discussion

Although numerous organizations, independent clinicians and scientists have alluded to the danger posed by the tremendous increase of infections caused by MDR bacteria, the research and development of new antibiotics is lagging far behind the ability of microorganisms to become resistant to current therapies (323, 324). Recent reports focused on the identification of agents being developed have shown that the number of antimicrobials in clinical trials is very low and that most of these compounds are active against Gram-positive pathogens (325, 313). These studies emphasize the danger posed by MDR Gram-negative bacteria since none of the therapies in development display a new mode of action (313, 325). The results reported here propose that severe outer membrane injury may represent a platform for novel drug development against Gram-negative bacteria.

Amphipathic AMPs have been previously shown to cause bacterial death via membrane disruption, but the therapeutic promise is hindered by their susceptibility to degradation, potential toxicity and high synthesis costs (326). A synthetic peptide, $_L(KLAKLAK)_2$, revealed high bactericidal activity combined with insignificant eukaryotic cell toxicity (289). Here we show that not only the all D-enantiomer of the $(KLAKLAK)_2$ peptide retains its antimicrobial activity against Gram-negative bacteria but is also resistant to degradation by the

CYP450 enzyme. We speculate that the ability to withstand proteolysis may allow lower therapeutic doses, longer availability and improved cost efficiency.

Despite the constant use of MIC to determine antimicrobial susceptibility and guide treatment, this method does not provide any insight into the equilibrium of the bacteria-drug interaction. Moreover, the MIC provides information exclusively about the susceptibility of free, actively growing organisms. The sensitivity of quiescent, slow growing bacteria or biofilms to antimicrobials remains unknown, and differs from the susceptibility of exponential phase organisms. Therefore, a large number of infections receive sub-inhibitory drug concentrations, potentially contributing to emergence of drug resistance. The *in vitro* dynamic interaction of $D(KLAKLAK)_2$ with several bacterial species showed that the number of bacteria decreases by two to four logs within 4 hours of treatment. Two commonly used *P. aeruginosa* strains, PAO1 and PA14, revealed different growth inhibition profiles. At MIC, PAO1 growth abruptly decreased within 4 hours, however, by 10 hours the rate of multiplication exceeded the peptide-induced killing rate. At concentrations higher than the MIC, we observed dose- and time-dependent growth inhibition. In contrast, PA14 was less sensitive to $D(KLAKLAK)_2$. Although the growth inhibition of PA14 was initially dose dependent, after 24 hours the overall peptide activity was not significant. In the case of *A. baumannii* 19606 and *E. coli* 25922, $D(KLAKLAK)_2$ induced dose- and time-dependent growth inhibition with cidal effect at all concentration after 10 hours and 24 hours, respectively. The differences in susceptibility to $D(KLAKLAK)_2$ may be attributed to several

factors: i) species- specificity, ii) already acquired resistance to antimicrobials, iii) growth conditions or iv) secretion of factors that may interfere with peptide activity. Most likely growth conditions play an important role. As mentioned before, MIC testing is performed under static conditions, whereas time-kill curves involve culture aeration that may greatly affect the growth rate. Another possibility is that drug sensitivity is altered by the type or stage of growth. In fact, our data indicated that the peptide is able to dismantle 24-hour-old biofilm grown on plastic plates. This raised the possibility that $D(KLAKLAK)_2$ is active not only against growing bacteria but also against quiescent cells or stationary phase bacteria. Notably, live-dead microscopy assays indicated that the peptide is active regardless of the growth stage. This observation is of crucial importance in the context of persistent infections. Most commonly used antibiotics interfere with protein, RNA, DNA, peptidoglycan and folic acid synthesis processes that are active only in growing bacteria (327). In contrast, persistent infections are caused by dormant or slow-growing organisms such those within biofilms. These infections are hardly eliminated by commonly used antibiotics and therefore more susceptible to acquiring resistance to antimicrobials.

To further distinguish the factors that influence $D(KLAKLAK)_2$ activity, we tested approximately 100 clinical isolates with different antibiotic susceptibilities. Our data indicated a lack of correlation between already acquired antimicrobial resistance and susceptibility to $D(KLAKLAK)_2$. Furthermore, combination therapy using piperacillin and $D(KLAKLAK)_2$, both at one half MIC, indicate a synergistic effect.

The mechanism of amphipathic AMPs action has been fairly well defined. Bacterial death occurs as a result of membrane permeabilization caused by peptide insertion into the lipid bilayer (268). Electron microscopy studies demonstrated membrane damage and formation of membrane blebs. Depending on the agents used, cell death and membrane deterioration occur at the same time or the membrane injury continues long after loss of cell viability. Similarly, we observed ultrastructural membrane damage starting at treatment with 37.5 µg/ml $D(KLAKLAK)_2$ and more obvious at 150 µg/ml peptide after 25 minutes of treatment. Using the lysozyme lysis assay, we showed that $D(KLAKLAK)_2$ induced a loss of membrane integrity as measured by the ability of the peptide to increase lysozyme access to its peptidoglycan substrate and induce cell lysis. Surprisingly, amounts as low as 20 µg/ml $D(KLAKLAK)_2$ created membrane pores large enough to allow complete cell lysis suggesting that the actual lipid bilayer damage is more intense than the modification observed in our electron micrographs.

As noted previously, bacterial membranes serve not only structural functions, but also support selective transport and energy storage (328). Membrane destabilization promotes loss of membrane potential, ultimately resulting in cell death. As shown by our results, $D(KLAKLAK)_2$ treatment induced dose-dependent membrane depolarization correlated with bacteria loss of viability.

As shown by our model membrane assays, $D(KLAKLAK)_2$ interacts solely with lipids specifically found in the bacterial membrane and this

interaction is inhibited by the addition of PC or cholesterol, components predominantly present in the eukaryotic membrane. Targeted activity allows the peptide to neutralize bacteria without perturbing the eukaryotic membrane. Indeed, concentrations of bactericidal concentrations of $D(KLAKLAK)_2$ had no hemolytic activity. In agreement, we previously showed that untargeted peptide could not induce apoptosis of KS1767 or MDA-MB-435 cells (309).

Taken together, our *in vitro* susceptibility and mechanistic studies suggest that $(KLAKLAK)_2$ maintains its activity against Gram-negative bacteria in the all-D proteolytically stable form. Natural and synthetic AMPs have been previously proposed as an alternative to antibiotics because their selective activity against microbes and lack of ability to quickly induce resistance (329). However, their high synthesis cost, instability, bioavailability and high sensitivity to salt and serum, and occasional toxicity have been considered hindering features for drug development (275). Although far from being ready for clinical applications, we speculate that a stable peptide may allow higher bioavailability, eliminate instability and improve cost-efficiency by using lower amounts of drug for treatment. In addition, our data indicated that the peptide activity is not influenced by either high salt or serum presence. Moreover, work performed in our laboratory exploiting the pro-apoptotic $D(KLAKLAK)_2$ capability revealed fairly low toxicity at therapeutic concentrations. However, we acknowledge that the toxicology profiles for cancer and infection may differ. Notably, the peptide alone or in combination with commonly used antibiotics is also active against persistent infections such as biofilms and MDR clinical isolates. In the event that

the peptide displays high toxicity at the concentrations necessary to eliminate infection *in vivo*, we propose that we could at least exploit this agent for combination therapy, as coating component for biomedical devices or as topical antibiotic.

In summary, we believe that the $D(KLAKLAK)_2$ mode of action may represent a stepping-stone for the development or improvement of drugs with broad activity against the lipid bilayer. On this note, a $D(KLAKLAK)_2$ variant with improved pro-apoptotic activity has already been reported (330). Therefore, our future experiments will focus on the engineering of divergent peptides with improved antibacterial activity, defining the peptide activity to eliminate infection *in vivo*, delineate its toxicological and pharmacokinetics profiles.

As previously stated, it is considered that development of resistance does not represent an obstacle for AMPs clinical development. In 2003, the FDA approved the lipopeptide antibiotic DAP. DAP is not, in its native form, a traditional AMP. However DAP renders itself a de-novo AMP in the presence of calcium ions. DAP is proposed to cause bacterial cell death in the same manner as traditional AMPs. It is widely accepted that DAP first binds to the Gram-positive bacterial membrane followed by rapid depolarization resulting in a loss of membrane potential which leads to protein, DNA and RNA synthesis inhibition, resulting in bacterial cell death.

Recently, Gram-positive strains with decreased susceptibility to DAP have been reported, yet the mechanism of resistance in enterococci remains unknown. Our findings indicated that, similar to *S. aureus*, development of DAP-

resistance in a vancomycin-resistant *E. faecalis* isolate is associated with alterations of the CW and CM properties. However, the genes linked to these changes in enterococci appear to be different from those described in *S. aureus*.

The treatment of modern-day enterococcal infections has become a major challenge since there is a lack of reliable options to treat ampicillin-resistant VRE infections. The off-label use of DAP is often employed in the treatment of severe enterococcal infections including VRE or those exhibiting high-level resistance to aminoglycosides. However, a major drawback for the successful use of this antibiotic is the emergence of resistance during DAP therapy. Moreover, enterococci are less susceptible *in vitro* to DAP than *S. aureus* with a breakpoint four-fold higher than for *S. aureus* ($\leq 4 \mu\text{g/ml}$ vs $\leq 1 \mu\text{g/ml}$, respectively) (CLSI document m100-s20). Thus, an understanding of the molecular events that lead to the development of DAP-resistance is of vital importance when designing new therapeutic strategies for salvaging the use of DAP against VRE (e.g., higher dose schemes or combination therapies) and has the potential to identify or validate new targets for the development of novel antimicrobial agents against these organisms.

An essential event for the activity of DAP is the calcium-mediated interaction of this antibiotic with the CM. The interaction of DAP with the CM of enterococci is not as well defined as the interaction of antimicrobial peptides with Gram-negative bacteria. Gram-positive cells have a thick layer of peptidoglycan that the antimicrobial peptide molecule must transverse to reach the target site of action. Therefore, modifications to this barrier that make the

peptide less able to interact with the membrane would reduce the effectiveness of the peptide. We identified marked distortions of the CW, surface and septal apparatus in the DAP-resistant isolate in our clinical strain pair set. Some of the cell surface changes (e.g., cell surface protrusions) have not been previously described with DAP resistance. These changes suggest that emergence of resistance to DAP in enterococci during therapy is also related to important alterations of CW homeostasis. Indeed, previous studies in *S. aureus* indicate that inhibition of peptidoglycan biosynthesis and activation of the CW stress response is involved in DAP action and non-susceptibility (331).

In addition, the surface charge appears to play an important role in the interaction of the antibiotic with the CM and it has been postulated that a more positively charged cell envelope can presumably “repel” cationic AMPs, including DAP, away from the CM and contribute to the development of resistance (317). A major contributor of the cell surface charge is the phospholipid composition of the inner and outer CM leaflets, such as the negatively charged cardiolipin (CL) and positively-charged amino derivatives of PG. In some *S. aureus* isolates, reduced susceptibility to DAP has been attributed to a decrease in the overall cell surface charge as a result of modifications in phospholipid content, mainly through an increased synthesis and translocation (“flipping”) of the positively charged lysyl-phosphatidylglycerol (L-PG) from the inner to the outer leaflet of the CM (317). Moreover, it has been shown that L-PG attenuates membrane perturbations caused by cationic antimicrobial peptides (332). Our findings suggest that, similar to *S. aureus*, development of DAP-

resistance in vancomycin-resistant *E. faecalis* is also associated with increased cell surface charge. However, unlike *S. aureus*, enterococci possess other amino-containing PL apart from L-PG including arginyl-, alanyl-, and diglucosyl-PG (333) which could also alter the CM charge or stabilize the CM and, thus, may contribute to the DAP-resistance phenotype.

Taken together, we have shown that the emergence of DAP resistance during therapy of VRE is associated with important alterations in DAP-induced CM potential and permeabilization with an increase in net cell surface charge. In addition, alterations of CM similar to those observed in *S. aureus* appear to play a role in DAP resistance in VRE. Our results suggest that the development of *in vivo* DAP resistance in VRE is associated with marked structural changes in the cell envelope with alterations in cell division and increased CW thickness.

Notably, the genetic strategy used by enterococci appears to be different from that described in *S. aureus* (personal communication, Cesar A. Arias, MD, PhD, University of Texas Medical School). Indeed, none of the genes previously associated with the emergence of DAP-resistance in *S. aureus* (319, 251) appeared mutated in the DAP-resistant isolate compared to its DAP-susceptible parental strain. Recent findings (unpublished, personal communication, Cesar A. Arias, MD, PhD, University of Texas Medical School) provide compelling evidence that changes in genes encoding CM phospholipid synthesis enzymes, namely CL synthase and glycerophosphoryl diesterphosphodiesterase in *E. faecalis* are important for development of DAP-resistance in enterococci. Findings (unpublished, personal communication, Cesar A. Arias, MD, PhD,

University of Texas Medical School) derived from genomic analysis of *E. faecalis* also identified unique mutations in the genes encoding a three component-regulatory system (LiaFRS, for *lipid-II* interacting *antibiotics*), not previously associated with DAP-resistance in *S. aureus*. This unique system is known to orchestrate the cell envelope response to antibiotics and antimicrobial peptides in some Gram-positive bacteria (the genes are present in most *Firmicutes*). The LiaFSR system has been well characterized in *B. subtilis* (334) *Streptococcus mutans* and pneumococci (335). In *B. subtilis* and *S. mutans*, the LiaFSR system is usually activated by the presence of antibiotics that disrupt CM (i.e., bacitracin, daptomycin, ramoplanin, nisin and vancomycin (334). In *B. subtilis*, LiaF is a membrane-anchored, negative regulator of LiaS (which is the sensor protein of the system and also functions as a histidine kinase that phosphorylates the response regulator, LiaR). LiaF interferes with the histidine kinase autophosphorylation by direct interactions, thereby suppressing activation of the cognate response regulator (LiaR) (336). Additionally, it is important to note that there may be several pathways for the development of DAP-resistance in enterococci, as shown in *S. aureus* (251, 337).

Taken together, we have shown that a synthetic AMP able to disrupt bacterial membrane non-specifically is active against Gram-negative organisms such as *P. aeruginosa*, *K. pneumoniae*, *E. coli* and *A. baumannii*, regardless of their antibiotic resistance profiles. We speculate that targeting the cell outer layer may represent a new strategy for antimicrobial development since the CM is essential for microbe integrity as well as energy storage. Although the scientific

community does not see development of resistance to AMPs as a concern, reduced sensitivity of *S. aureus* and *E. faecalis* to de-novo AMP, DAP, emerged as a result of treatment. Resistance associated with DAP include, most importantly, modifications to the thick peptidoglycan layer surrounding the CM of Gram-positive organisms. Since this is a Gram-positive specific cell feature, the mechanism of resistance related to changes in the peptidoglycan layer is also specific to Gram-positive cells.

In summary, the detailed analysis of the mode of action of the AMP_D(KLAKLAK)₂ with lipid bilayers may represent a stepping-stone for the development or improvement of drugs with broad activity against in Gram-negative bacterial membrane. We also believe that type of DAP resistance acquired by enterococci and staphylococci will not affect the treatment with AMP_D(KLAKLAK)₂ because resistance to DAP in these organisms is related to Gram-positive specific cell features such as the cell-wall.

Chapter 6: References

1. Kirby, W. M. 1944. Extraction of a Highly Potent Penicillin Inactivator from Penicillin Resistant Staphylococci. *Science* 99:452-453.
2. Spink, W. W., and V. Ferris. 1945. QUANTITATIVE ACTION OF PENICILLIN INHIBITOR FROM PENICILLIN-RESISTANT STRAINS OF STAPHYLOCOCCI. *Science* 102:221-223.
3. Bondi, A., Jr., and C. C. Dietz. 1945. Penicillin resistant staphylococci. *Proc Soc Exp Biol Med* 60:55-58.
4. Dzidic, S., J. Suskovic, and B. Kos. 2008. Antibiotic Resistance Mechanisms in Bacteria: Biochemical and Genetic Aspect. *Food Technology and Biotechnology* 46.
5. Krasovec, R., and I. Jerman. 2003. Bacterial multicellularity as a possible source of antibiotic resistance. *Med Hypotheses* 60:484-488.
6. Woodford, N., and M. J. Ellington. 2007. The emergence of antibiotic resistance by mutation. *Clinical Microbiology and Infection* 13:5-18.
7. Martinez, J. L., and F. Baquero. 2000. Mutation Frequencies and Antibiotic Resistance. *Antimicrob. Agents Chemother.* 44:1771-1777.
8. Ruiz, J. 2003. Mechanisms of resistance to quinolones: target alterations, decreased accumulation and DNA gyrase protection. *Journal of Antimicrobial Chemotherapy* 51:1109-1117.

9. Depardieu, F., I. Podglajen, R. Leclercq, E. Collatz, and P. Courvalin. 2007. Modes and Modulations of Antibiotic Resistance Gene Expression. *Clin. Microbiol. Rev.* 20:79-114.
10. Piddock, L. J. V. 2006. Clinically Relevant Chromosomally Encoded Multidrug Resistance Efflux Pumps in Bacteria. *Clin. Microbiol. Rev.* 19:382-402.
11. Wolter, D. J., N. D. Hanson, and P. D. Lister. 2004. Insertional inactivation of *oprD* in clinical isolates of *Pseudomonas aeruginosa* leading to carbapenem resistance. *FEMS Microbiology Letters* 236:137-143.
12. Oliver, A., F. Baquero, and J. Blázquez. 2002. The mismatch repair system (*mutS*, *mutL* and *uvrD* genes) in *Pseudomonas aeruginosa*: molecular characterization of naturally occurring mutants. *Molecular Microbiology* 43:1641-1650.
13. Rayssiguier, C., D. S. Thaler, and M. Radman. 1989. The barrier to recombination between *Escherichia coli* and *Salmonella typhimurium* is disrupted in mismatch-repair mutants. *Nature* 342:396-401.
14. Giraud, A., I. Matic, M. Radman, M. Fons, and F. Taddei. 2002. Mutator Bacteria as a Risk Factor in Treatment of Infectious Diseases. *Antimicrob. Agents Chemother.* 46:863-865.
15. Blazquez, J. 2003. Hypermutation as a factor contributing to the acquisition of antimicrobial resistance. *Clin Infect Dis* 37:1201-1209.
16. Griffith, F. 1928. The Significance of Pneumococcal Types. *J Hyg (Lond)* 27:113-159.

17. Thomas, C. M., and K. M. Nielsen. 2005. Mechanisms of, and Barriers to, Horizontal Gene Transfer between Bacteria. *Nat Rev Micro* 3:711-721.
18. THOMAS ALBRECHT, P. 1996. *Medical Microbiology*, 4th edition. University of Texas Medical Branch at Galveston, Galveston.
19. Wright, G. D. 2005. Bacterial resistance to antibiotics: Enzymatic degradation and modification. *Advanced Drug Delivery Reviews* 57:1451-1470.
20. Lambert, P. A. 2005. Bacterial resistance to antibiotics: Modified target sites. *Advanced Drug Delivery Reviews* 57:1471-1485.
21. Kumar, A., and H. P. Schweizer. 2005. Bacterial resistance to antibiotics: Active efflux and reduced uptake. *Advanced Drug Delivery Reviews* 57:1486-1513.
22. Mobashery, S., and E. F. A. Jr. 2001. *Bacterial Antibiotic Resistance*. John Wiley & Sons, Ltd.
23. Kim, Y.-H., C.-J. Cha, and C. E. Cerniglia. 2002. Purification and characterization of an erythromycin esterase from an erythromycin-resistant *Pseudomonas* sp. *FEMS Microbiology Letters* 210:239-244.
24. Nakamura, A, Nakazawa, K, Miyakozawa, I, Mizukoshi, S, Tsurubuchi, Nakagawa, M, O'Hara, Sawai, and T. 2000. Macrolide esterase-producing *Escherichia coli* clinically isolated in Japan. Nature Publishing Group, Basingstoke, ROYAUME-UNI.
25. Fillgrove, K. L., S. Pakhomova, M. E. Newcomer, and R. N. Armstrong. 2003. Mechanistic Diversity of Fosfomycin Resistance in Pathogenic Microorganisms. *Journal of the American Chemical Society* 125:15730-15731.

26. Wangrong, Y., Moore, F. Ian, Koteva, P. Kalinka, Bareich, C. David, Hughes, W. Donald, Wright, and D. Gerard. 2004. TetX is a flavin-dependent monooxygenase conferring resistance to tetracycline antibiotics. American Society for Biochemistry and Molecular Biology, Bethesda, MD, ETATS-UNIS.
27. Nagai, K., T. A. Davies, M. R. Jacobs, and P. C. Appelbaum. 2002. Effects of Amino Acid Alterations in Penicillin-Binding Proteins (PBPs) 1a, 2b, and 2x on PBP Affinities of Penicillin, Ampicillin, Amoxicillin, Cefditoren, Cefuroxime, Cefprozil, and Cefaclor in 18 Clinical Isolates of Penicillin-Susceptible, -Intermediate, and -Resistant Pneumococci. *Antimicrob. Agents Chemother.* 46:1273-1280.
28. Dowson, C. G., T. J. Coffey, and B. G. Spratt. 1994. Origin and molecular epidemiology of penicillin-binding-protein-mediated resistance to [beta]-lactam antibiotics. *Trends in Microbiology* 2:361-366.
29. Tenover, F. C. 2006. Mechanisms of antimicrobial resistance in bacteria. *American journal of infection control* 34:S3-S10.
30. Weisblum, B. 1995. Erythromycin resistance by ribosome modification. *Antimicrob. Agents Chemother.* 39:577-585.
31. Khodursky, A. B., E. L. Zechiedrich, and N. R. Cozzarelli. 1995. Topoisomerase IV is a target of quinolones in Escherichia coli. *Proceedings of the National Academy of Sciences* 92:11801-11805.
32. Zgurskaya, H. I., and H. Nikaido. 1999. Bypassing the periplasm: Reconstitution of the AcrAB multidrug efflux pump of Escherichia coli. *Proceedings of the National Academy of Sciences* 96:7190-7195.

33. Webber, M. A., and L. J. V. Piddock. 2003. The importance of efflux pumps in bacterial antibiotic resistance. *Journal of Antimicrobial Chemotherapy* 51:9-11.
34. Bolhuis, H., H. W. van Veen, B. Poolman, A. J. M. Driessen, and W. N. Konings. 1997. Mechanisms of multidrug transporters. *FEMS Microbiology Reviews* 21:55-84.
35. Nikaido, H. 2003. Molecular Basis of Bacterial Outer Membrane Permeability Revisited. *Microbiol. Mol. Biol. Rev.* 67:593-656.
36. Denyer, S. P., and J. Y. Maillard. 2002. Cellular impermeability and uptake of biocides and antibiotics in Gram-negative bacteria. *Journal of Applied Microbiology* 92:35S-45S.
37. Chevalier, J., J.-M. Pagès, and M. Mallea. 1999. In Vivo Modification of Porin Activity Conferring Antibiotic Resistance to *Enterobacter aerogenes*. *Biochemical and Biophysical Research Communications* 266:248-251.
38. Dé, E., A. Baslé, M. Jaquinod, N. Saint, M. Mallea, G. Molle, and J.-M. Pagès. 2001. A new mechanism of antibiotic resistance in *Enterobacteriaceae* induced by a structural modification of the major porin. *Molecular Microbiology* 41:189-198.
39. Hancock, R. E. W., and F. S. L. Brinkman. 2002. FUNCTION OF *PSEUDOMONAS* PORINS IN UPTAKE AND EFFLUX. *Annual Review of Microbiology* 56:17-38.
40. Warburton, D. W., B. Bowen, and A. Konkle. 1994. The survival and recovery of *Pseudomonas aeruginosa* and its effect upon salmonellae in water: methodology to test bottled water in Canada. *Canadian Journal of Microbiology* 40:987-992.

41. Favero, M. S., L. A. Carson, W. W. Bond, and N. J. Petersen. 1971. *Pseudomonas aeruginosa*: Growth in Distilled Water from Hospitals. *Science* 173:836-838.
42. Giamarellou, H. 2002. Prescribing guidelines for severe *Pseudomonas* infections. *J Antimicrob Chemother* 49:229-233.
43. Pollack, M., N. S. Taylor, and L. T. Callahan, 3rd. 1977. Exotoxin production by clinical isolates of *pseudomonas aeruginosa*. *Infect. Immun.* 15:776-780.
44. Somerville, G., C. A. Mikoryak, and L. Reitzer. 1999. Physiological Characterization of *Pseudomonas aeruginosa* during Exotoxin A Synthesis: Glutamate, Iron Limitation, and Aconitase Activity. *J. Bacteriol.* 181:1072-1078.
45. Kadurugamuwa, J. L., and T. J. Beveridge. 1997. Natural release of virulence factors in membrane vesicles by *Pseudomonas aeruginosa* and the effect of aminoglycoside antibiotics on their release. *Journal of Antimicrobial Chemotherapy* 40:615-621.
46. Raivio, T. L., E. E. Ujack, H. R. Rabin, and D. G. Storey. 1994. Association between transcript levels of the *Pseudomonas aeruginosa* *regA*, *regB*, and *toxA* genes in sputa of cystic fibrosis patients. *Infect. Immun.* 62:3506-3514.
47. Hancock, R. E., L. M. Mutharia, L. Chan, R. P. Darveau, D. P. Speert, and G. B. Pier. 1983. *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis: a class of serum-sensitive, nontypable strains deficient in lipopolysaccharide O side chains. *Infect. Immun.* 42:170-177.
48. Pier, G. B., G. Meluleni, and E. Neuger. 1992. A murine model of chronic mucosal colonization by *Pseudomonas aeruginosa*. *Infect. Immun.* 60:4768-4776.

49. Mahenthiralingam, E., M. E. Campbell, and D. P. Speert. 1994. Nonmotility and phagocytic resistance of *Pseudomonas aeruginosa* isolates from chronically colonized patients with cystic fibrosis. *Infect. Immun.* 62:596-605.
50. Boucher, J. C., H. Yu, M. H. Mudd, and V. Deretic. 1997. Mucoid *Pseudomonas aeruginosa* in cystic fibrosis: characterization of muc mutations in clinical isolates and analysis of clearance in a mouse model of respiratory infection. *Infect. Immun.* 65:3838-3846.
51. Arora, S. K., B. W. Ritchings, E. C. Almira, S. Lory, and R. Ramphal. 1998. The *Pseudomonas aeruginosa* Flagellar Cap Protein, FliD, Is Responsible for Mucin Adhesion. *Infect. Immun.* 66:1000-1007.
52. Gewirtz, A. T., T. A. Navas, S. Lyons, P. J. Godowski, and J. L. Madara. 2001. Cutting Edge: Bacterial Flagellin Activates Basolaterally Expressed TLR5 to Induce Epithelial Proinflammatory Gene Expression. *The Journal of Immunology* 167:1882-1885.
53. O'Toole, G. A., and R. Kolter. 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Molecular Microbiology* 30:295-304.
54. O'Toole, G. A., and R. Kolter. 1998. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Molecular Microbiology* 28:449-461.
55. Yahr, T. L., L. M. Mende-Mueller, M. B. Friese, and D. W. Frank. 1997. Identification of type III secreted products of the *Pseudomonas aeruginosa* exoenzyme S regulon. *J. Bacteriol.* 179:7165-7168.

56. Coburn, B., I. Sekirov, and B. B. Finlay. 2007. Type III Secretion Systems and Disease. *Clin. Microbiol. Rev.* 20:535-549.
57. Veessenmeyer, J. L., A. R. Hauser, T. Lisboa, and J. Rello. 2009. *Pseudomonas aeruginosa* virulence and therapy: Evolving translational strategies *. *Critical Care Medicine* 37:1777-1786 1710.1097/CCM.1770b1013e31819ff31137.
58. Feltman, H., G. Schulert, S. Khan, M. Jain, L. Peterson, and A. R. Hauser. 2001. Prevalence of type III secretion genes in clinical and environmental isolates of *Pseudomonas aeruginosa*. *Microbiology* 147:2659-2669.
59. Sun, J., and J. T. Barbieri. 2004. ExoS Rho GTPase-activating Protein Activity Stimulates Reorganization of the Actin Cytoskeleton through Rho GTPase Guanine Nucleotide Disassociation Inhibitor. *Journal of Biological Chemistry* 279:42936-42944.
60. Sato, H., and D. W. Frank. 2004. ExoU is a potent intracellular phospholipase. *Molecular Microbiology* 53:1279-1290.
61. Phillips, R. M., D. A. Six, E. A. Dennis, and P. Ghosh. 2003. In Vivo Phospholipase Activity of the *Pseudomonas aeruginosa* Cytotoxin ExoU and Protection of Mammalian Cells with Phospholipase A2 Inhibitors. *Journal of Biological Chemistry* 278:41326-41332.
62. Rabin, S. D. P., J. L. Veessenmeyer, K. T. Bieging, and A. R. Hauser. 2006. A C-Terminal Domain Targets the *Pseudomonas aeruginosa* Cytotoxin ExoU to the Plasma Membrane of Host Cells. *Infect. Immun.* 74:2552-2561.
63. Saliba, A. M., D. O. Nascimento, M. C. A. Silva, M. C. Assis, C. R. M. Gayer, B. Raymond, M. G. P. Coelho, E. A. Marques, L. Touqui, R. M. Albano, U. G.

- Lopes, D. D. Paiva, P. T. Bozza, and M. C. Plotkowski. 2005. Eicosanoid-mediated proinflammatory activity of *Pseudomonas aeruginosa* ExoU. *Cellular Microbiology* 7:1811-1822.
64. Juhas, M., L. Eberl, and B. Tümmler. 2005. Quorum sensing: the power of cooperation in the world of *Pseudomonas*. *Environmental Microbiology* 7:459-471.
65. Ochsner, U. A., A. Fiechter, and J. Reiser. 1994. Isolation, characterization, and expression in *Escherichia coli* of the *Pseudomonas aeruginosa* rhlAB genes encoding a rhamnosyltransferase involved in rhamnolipid biosurfactant synthesis. *Journal of Biological Chemistry* 269:19787-19795.
66. Ochsner, U. A., A. K. Koch, A. Fiechter, and J. Reiser. 1994. Isolation and characterization of a regulatory gene affecting rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. *J. Bacteriol.* 176:2044-2054.
67. Latifi, A., M. K. Winson, M. Foglino, B. W. Bycroft, G. S. A. B. Stewart, A. Lazdunski, and P. Williams. 1995. Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in *Pseudomonas aeruginosa* PAO1. *Molecular Microbiology* 17:333-343.
68. Winson, M. K., M. Camara, A. Latifi, M. Foglino, S. R. Chhabra, M. Daykin, M. Bally, V. Chapon, G. P. Salmond, and B. W. Bycroft. 1995. Multiple N-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences* 92:9427-9431.

69. Brint, J. M., and D. E. Ohman. 1995. Synthesis of multiple exoproducts in *Pseudomonas aeruginosa* is under the control of RhlR-RhlI, another set of regulators in strain PAO1 with homology to the autoinducer-responsive LuxR-LuxI family. *J. Bacteriol.* 177:7155-7163.
70. Chapon-Hervé, V., M. Akrim, A. Latifi, P. Williams, A. Lazdunski, and M. Bally. 1997. Regulation of the xcp secretion pathway by multiple quorum-sensing modulons in *Pseudomonas aeruginosa*. *Molecular Microbiology* 24:1169-1178.
71. Schuster, M., C. P. Lostroh, T. Ogi, and E. P. Greenberg. 2003. Identification, Timing, and Signal Specificity of *Pseudomonas aeruginosa* Quorum-Controlled Genes: a Transcriptome Analysis. *J. Bacteriol.* 185:2066-2079.
72. Rossolini, G. M., and E. Mantengoli. 2005. Treatment and control of severe infections caused by multiresistant *Pseudomonas aeruginosa*. *Clin Microbiol Infect* 11 Suppl 4:17-32.
73. Ambler, R. P. 1980. The Structure of {beta}-Lactamases. *Philosophical Transactions of the Royal Society of London. B, Biological Sciences* 289:321-331.
74. Bush, K, Jacoby, A. G, Medeiros, and A. A. 1995. A functional classification scheme for β -lactamases and its correlation with molecular structure. American Society for Microbiology, Washington, DC, ETATS-UNIS.
75. Langaee, T. Y., L. Gagnon, and A. Huletsky. 2000. Inactivation of the ampD Gene in *Pseudomonas aeruginosa* Leads to Moderate-Basal-Level and

- Hyperinducible AmpC beta -Lactamase Expression. Antimicrob. Agents Chemother. 44:583-589.
76. Bagge, N., O. Ciofu, M. Hentzer, J. I. A. Campbell, M. Givskov, and N. Hoiby. 2002. Constitutive High Expression of Chromosomal {beta}-Lactamase in *Pseudomonas aeruginosa* Caused by a New Insertion Sequence (IS1669) Located in ampD. Antimicrob. Agents Chemother. 46:3406-3411.
 77. Bert, F. d. r., C. Branger, and N. Lambert-Zechovsky. 2002. Identification of PSE and OXA (E)-lactamase genes in *Pseudomonas aeruginosa* using PCR,Ärestriction fragment length polymorphism. Journal of Antimicrobial Chemotherapy 50:11-18.
 78. Nordmann, P., and M. Guibert. 1998. Extended-spectrum beta-lactamases in *Pseudomonas aeruginosa*. The Journal of antimicrobial chemotherapy 42:128-131.
 79. Naas, T, Nordmann, and P. 1999. OXA-type {beta}-lactamases. Bentham, Sharjah, EMIRATS ARABES UNIS.
 80. Livermore, D. M. 1984. Penicillin-Binding Proteins, Porins Andouter-Membrane Permeability of Carbenicillin-Resistant and -Susceptible Strains of *Pseudomonas Aeruginosa*. J Med Microbiol 18:261-270.
 81. Livermore, D. M. 2001. Of *Pseudomonas*, porins, pumps and carbapenems. Journal of Antimicrobial Chemotherapy 47:247-250.
 82. Livermore, D. M. 2002. Multiple Mechanisms of Antimicrobial Resistance in *Pseudomonas aeruginosa*: Our Worst Nightmare? Clinical Infectious Diseases 34:634-640.

83. Kohler, T., M. Michea-Hamzehpour, S. F. Epp, and J.-C. Pechere. 1999. Carbapenem Activities against *Pseudomonas aeruginosa*: Respective Contributions of OprD and Efflux Systems. *Antimicrob. Agents Chemother.* 43:424-427.
84. Vakulenko, S. B., and S. Mobashery. 2003. Versatility of Aminoglycosides and Prospects for Their Future. *Clin. Microbiol. Rev.* 16:430-450.
85. Poole, K. 2005. Aminoglycoside Resistance in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 49:479-487.
86. Magnet, S., and J. S. Blanchard. 2004. Molecular Insights into Aminoglycoside Action and Resistance. *Chemical Reviews* 105:477-498.
87. Haddad, J., L. P. Kotra, B. Llano-Sotelo, C. Kim, E. F. Azucena, M. Liu, S. B. Vakulenko, C. S. Chow, and S. Mobashery. 2002. Design of Novel Antibiotics that Bind to the Ribosomal Acyltransfer Site. *Journal of the American Chemical Society* 124:3229-3237.
88. Doi, Y., and Y. Arakawa. 2007. 16S Ribosomal RNA Methylation: Emerging Resistance Mechanism against Aminoglycosides. *Clinical Infectious Diseases* 45:88-94.
89. Hooper, D. C. 2001. Mechanisms of Action of Antimicrobials: Focus on Fluoroquinolones. *Clinical Infectious Diseases* 32:S9-S15.
90. Seifert, H., R. Baginski, A. Schulze, and G. Pulverer. 1993. Antimicrobial susceptibility of *Acinetobacter* species. *Antimicrob. Agents Chemother.* 37:750-753.

91. Seifert, H., A. Schulze, R. Baginski, and G. Pulverer. 1994. Comparison of four different methods for epidemiologic typing of *Acinetobacter baumannii*. *J. Clin. Microbiol.* 32:1816-1819.
92. Berlau, J., H. Aucken, H. Malnick, and T. Pitt. 1999. Distribution of *Acinetobacter* Species on Skin of Healthy Humans. *European Journal of Clinical Microbiology & Infectious Diseases* 18:179-183.
93. Seifert, H., L. Dijkshoorn, P. Gerner-Smidt, N. Pelzer, I. Tjernberg, and M. Vaneechoutte. 1997. Distribution of *Acinetobacter* species on human skin: comparison of phenotypic and genotypic identification methods. *J. Clin. Microbiol.* 35:2819-2825.
94. Gaynes, Robert, Edwards, and R. Jonathan. 2005. Overview of nosocomial infections caused by gram-negative bacilli. Oxford University Press, Oxford, ROYAUME-UNI.
95. Anstey, N. M., B. J. Currie, M. Hassell, D. Palmer, B. Dwyer, and H. Seifert. 2002. Community-Acquired Bacteremic *Acinetobacter* Pneumonia in Tropical Australia Is Caused by Diverse Strains of *Acinetobacter baumannii*, with Carriage in the Throat in At-Risk Groups. *J. Clin. Microbiol.* 40:685-686.
96. Anstey, N. M., B. J. Currie, and K. M. Withnall. 1992. Community-Acquired *Acinetobacter* Pneumonia in the Northern Territory of Australia. *Clinical Infectious Diseases* 14:83-91.
97. Bick, J. A., and J. D. Semel. 1993. Fulminant Community-Acquired *Acinetobacter* Pneumonia in a Healthy Woman. *Clinical Infectious Diseases* 17:820-821.

98. Gottlieb, T., and D. J. Barnes. 1989. COMMUNITY-ACQUIRED ACINETOBACTER PNEUMONIA. Australian and New Zealand Journal of Medicine 19:259-260.
99. Leung, W.-S., C.-M. Chu, K.-Y. Tsang, F.-H. Lo, K.-F. Lo, and P.-L. Ho. 2006. Fulminant Community-Acquired Acinetobacter baumannii Pneumonia as a Distinct Clinical Syndrome*. Chest 129:102-109.
100. Wisplinghoff, H., T. Bischoff, S. M. Tallent, H. Seifert, R. P. Wenzel, and M. B. Edmond. 2004. Nosocomial Bloodstream Infections in US Hospitals: Analysis of 24,179 Cases from a Prospective Nationwide Surveillance Study. Clinical Infectious Diseases 39:309-317.
101. Seifert, H., A. Strate, and G. Pulverer. 1995. Nosocomial Bacteremia due to Acinetobacter baumannii: Clinical Features, Epidemiology, and Predictors of Mortality. Medicine 74:340-349.
102. Metan, G., E. Alp, B. Aygen, and B. Sumerkan. 2007. Acinetobacter baumannii meningitis in post-neurosurgical patients: clinical outcome and impact of carbapenem resistance. Journal of Antimicrobial Chemotherapy 60:197-199.
103. Olut, A. I., and E. Erkek. 2005. Early prosthetic valve endocarditis due to Acinetobacter baumannii: A case report and brief review of the literature. Scandinavian Journal of Infectious Diseases 37:919-921.
104. Rizos, Ioannis, Tsiodras, Sotirios, Papathanasiou, Rigopoulos, Angelos, Barbetseas, John, Stefanadis, and Christodoulos. 2007. Prosthetic valve endocarditis due to Acinetobacter spp : A rare case and literature review. Lippincott Williams & Wilkins, Hagerstown, MD, ETATS-UNIS.

105. Starakis, I., A. Blikas, D. Siagris, M. Marangos, C. Karatza, and H. Bassaris. 2006. Prosthetic Valve Endocarditis Caused by *Acinetobacter lwoffii*: A Case Report and Review. *Cardiology in Review* 14:45-49.
106. Valero, C., M. C. Farias, D. Garcia Palomo, J. C. Mazarrasa, and J. Gonzalez Macias. 1999. Endocarditis due to *Acinetobacter lwoffii* on native mitral valve. *International journal of cardiology* 69:97-99.
107. Corrigan, K. M., N. Y. Harmis, and M. D. P. Willcox. 2001. Association of *Acinetobacter* Species With Contact Lens-induced Adverse Responses. *Cornea* 20:463-466.
108. Kau, H.-C., C.-C. Tsai, S.-C. Kao, W.-M. Hsu, and J.-H. Liu. 2002. Corneal ulcer of the side port after phacoemulsification induced by *Acinetobacter baumannii*. *Journal of cataract and refractive surgery* 28:895-897.
109. Vallenet, D., P. Nordmann, V. r. Barbe, L. Poirel, S. Mangenot, E. Bataille, C. Dossat, S. Gas, A. Kreimeyer, P. Lenoble, S. Oztas, J. Poulain, B. a. Segurens, C. Robert, C. Abergel, J.-M. Claverie, D. Raoult, C. Modigue, J. Weissenbach, and S. Cruveiller. 2008. Comparative Analysis of *Acinetobacters*: Three Genomes for Three Lifestyles. *PLoS ONE* 3:e1805.
110. Gordon, N. C., and D. W. Wareham. Multidrug-resistant *Acinetobacter baumannii*: mechanisms of virulence and resistance. *International journal of antimicrobial agents* 35:219-226.
111. Lee, H. W., Y. M. Koh, J. Kim, J. C. Lee, Y. C. Lee, S. Y. Seol, and D. T. Cho. 2008. Capacity of multidrug-resistant clinical isolates of *Acinetobacter*

- baumannii to form biofilm and adhere to epithelial cell surfaces. *Clinical Microbiology and Infection* 14:49-54.
112. Vidal, R, Dominguez, M, Urrutia, H, Bello, Gonzalez, G, Garcia, A, and Zemelman. 1996. Biofilm formation by *Acinetobacter baumannii*. Faculty Press, Cambridge, ROYAUME-UNI.
 113. Choi, C. H., S. H. Hyun, J. Y. Lee, J. S. Lee, Y. S. Lee, S. A. Kim, J.-P. Chae, S. M. Yoo, and J. C. Lee. 2008. *Acinetobacter baumannii* outer membrane protein A targets the nucleus and induces cytotoxicity. *Cellular Microbiology* 10:309-319.
 114. Bou, G., G. Cervero, M. A. Dominguez, C. Quereda, and J. Martinez-Beltran. 2000. Characterization of a Nosocomial Outbreak Caused by a Multiresistant *Acinetobacter baumannii* Strain with a Carbapenem-Hydrolyzing Enzyme: High-Level Carbapenem Resistance in *A. baumannii* Is Not Due Solely to the Presence of beta -Lactamases. *J. Clin. Microbiol.* 38:3299-3305.
 115. Hujer, K. M., A. M. Hujer, E. A. Hulten, S. Bajaksouzian, J. M. Adams, C. J. Donskey, D. J. Ecker, C. Massire, M. W. Eshoo, R. Sampath, J. M. Thomson, P. N. Rather, D. W. Craft, J. T. Fishbain, A. J. Ewell, M. R. Jacobs, D. L. Paterson, and R. A. Bonomo. 2006. Analysis of Antibiotic Resistance Genes in Multidrug-Resistant *Acinetobacter* sp. Isolates from Military and Civilian Patients Treated at the Walter Reed Army Medical Center. *Antimicrob. Agents Chemother.* 50:4114-4123.
 116. Perilli, M., A. Felici, A. Oratore, G. Cornaglia, G. Bonfiglio, G. M. Rossolini, and G. Amicosante. 1996. Characterization of the chromosomal

- cephalosporinases produced by *Acinetobacter lwoffii* and *Acinetobacter baumannii* clinical isolates. *Antimicrob. Agents Chemother.* 40:715-719.
117. Ruiz, M., S. Marti, F. Fernandez-Cuenca, A. Pascual, and J. Vila. 2007. High prevalence of carbapenem-hydrolysing oxacillinases in epidemiologically related and unrelated *Acinetobacter baumannii* clinical isolates in Spain. *Clinical Microbiology and Infection* 13:1192-1198.
 118. Abbo, A., Y. Carmeli, S. Navon-Venezia, Y. Siegman-Igra, and M. Schwaber. 2007. Impact of multi-drug-resistant *Acinetobacter baumannii* on clinical outcomes. *European Journal of Clinical Microbiology & Infectious Diseases* 26:793-800.
 119. Albrecht, M. A., M. E. Griffith, C. K. Murray, K. K. Chung, E. E. Horvath, J. A. Ward, D. R. Hospenthal, J. B. Holcomb, and S. E. Wolf. 2006. Impact of *Acinetobacter* Infection on the Mortality of Burn Patients. *Journal of the American College of Surgeons* 203:546-550.
 120. H  ritier, C., L. Poirel, and P. Nordmann. 2006. Cephalosporinase over-expression resulting from insertion of ISAbal in *Acinetobacter baumannii*. *Clinical Microbiology and Infection* 12:123-130.
 121. Segal, H., E. C. Nelson, and B. G. Elisha. 2004. Genetic Environment and Transcription of ampC in an *Acinetobacter baumannii* Clinical Isolate. *Antimicrob. Agents Chemother.* 48:612-614.
 122. Heritier, C., L. Poirel, D. Aubert, and P. Nordmann. 2003. Genetic and Functional Analysis of the Chromosome-Encoded Carbapenem-Hydrolyzing

- Oxacillinase OXA-40 of *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 47:268-273.
123. Brown, S., and S. G. B. Amyes. 2005. The sequences of seven class D β -lactamases isolated from carbapenem-resistant *Acinetobacter baumannii* from four continents. *Clinical Microbiology and Infection* 11:326-329.
 124. Poirel, L., and P. Nordmann. 2006. Carbapenem resistance in *Acinetobacter baumannii*: mechanisms and epidemiology. *Clinical Microbiology and Infection* 12:826-836.
 125. Queenan, A. M., and K. Bush. 2007. Carbapenemases: the Versatile β -Lactamases. *Clin. Microbiol. Rev.* 20:440-458.
 126. Walsh, T. R., M. A. Toleman, L. Poirel, and P. Nordmann. 2005. Metallo- β -Lactamases: the Quiet before the Storm? *Clin. Microbiol. Rev.* 18:306-325.
 127. Costa, S. F., J. Woodcock, M. Gill, R. Wise, A. A. Barone, H. Caiaffa, and A. S. S. Levin. 2000. Outer-membrane proteins pattern and detection of β -lactamases in clinical isolates of imipenem-resistant *Acinetobacter baumannii* from Brazil. *International journal of antimicrobial agents* 13:175-182.
 128. Tomas, D. Maria, Beceiro, Alejandro, Perez, Astrid, Velasco, David, Moure, Rita, Villanueva, Rosa, B. Martinez, Jesus, Bou, and German. 2005. Cloning and functional analysis of the gene encoding the 33- to 36-kilodalton outer membrane protein associated with carbapenem resistance in *Acinetobacter baumannii*. American Society for Microbiology, Washington, DC, ETATS-UNIS.
 129. Fernandez-Cuenca, F., L. Martinez-Martinez, M. C. Conejo, J. A. Ayala, E. J. Perea, and A. Pascual. 2003. Relationship between β -lactamase production,

- outer membrane protein and penicillin-binding protein profiles on the activity of carbapenems against clinical isolates of *Acinetobacter baumannii*. *Journal of Antimicrobial Chemotherapy* 51:565-574.
130. Higgins, P. G., H. Wisplinghoff, D. Stefanik, and H. Seifert. 2004. Selection of topoisomerase mutations and overexpression of *adeB* mRNA transcripts during an outbreak of *Acinetobacter baumannii*. *Journal of Antimicrobial Chemotherapy* 54:821-823.
 131. Magnet, S., P. Courvalin, and T. Lambert. 2001. Resistance-Nodulation-Cell Division-Type Efflux Pump Involved in Aminoglycoside Resistance in *Acinetobacter baumannii* Strain BM4454. *Antimicrob. Agents Chemother.* 45:3375-3380.
 132. Fernandez-Cuenca, F., L. Martiinez-Martinez, M. C. Conejo, J. A. Ayala, E. J. Perea, and A. Pascual. 2003. Relationship between β -lactamase production, outer membrane protein and penicillin-binding protein profiles on the activity of carbapenems against clinical isolates of *Acinetobacter baumannii*. *Journal of Antimicrobial Chemotherapy* 51:565-574.
 133. Obara, M., and T. Nakae. 1991. Mechanisms of resistance to β -lactam antibiotics in *Acinetobacter calcoaceticus*. *Journal of Antimicrobial Chemotherapy* 28:791-800.
 134. Siroy, A., P. Cosette, D. Seyer, C. Lemtre-Guillier, D. Vallenet, A. Van Dorsselaer, S. Boyer-Mariotte, T. Jouenne, and E. De. 2006. Global Comparison of the Membrane Subproteomes between a Multidrug-Resistant *Acinetobacter*

- baumannii Strain and a Reference Strain. *Journal of Proteome Research* 5:3385-3398.
135. Nemec, A., L. Dolzani, S. Brisse, P. van den Broek, and L. Dijkshoorn. 2004. Diversity of aminoglycoside-resistance genes and their association with class 1 integrons among strains of pan-European *Acinetobacter baumannii* clones. *J Med Microbiol* 53:1233-1240.
136. Hamouda, A., and S. G. B. Amyes. 2004. Novel *gyrA* and *parC* point mutations in two strains of *Acinetobacter baumannii* resistant to ciprofloxacin. *Journal of Antimicrobial Chemotherapy* 54:695-696.
137. Seward, R. J., T. Lambert, and K. J. Towner. 1998. Molecular epidemiology of aminoglycoside resistance in *Acinetobacter* spp. *J Med Microbiol* 47:455-462.
138. Su, X.-Z., J. Chen, T. Mizushima, T. Kuroda, and T. Tsuchiya. 2005. AbeM, an H⁺-Coupled *Acinetobacter baumannii* Multidrug Efflux Pump Belonging to the MATE Family of Transporters. *Antimicrob. Agents Chemother.* 49:4362-4364.
139. Guardabassi, L., L. Dijkshoorn, J. M. Collard, J. E. Olsen, and A. Dalsgaard. 2000. Distribution and in-vitro transfer of tetracycline resistance determinants in clinical and aquatic *Acinetobacter* strains. *J Med Microbiol* 49:929-936.
140. Ribera, A., I. Roca, J. Ruiz, I. Gibert, and J. Vila. 2003. Partial characterization of a transposon containing the *tet(A)* determinant in a clinical isolate of *Acinetobacter baumannii*. *Journal of Antimicrobial Chemotherapy* 52:477-480.
141. Gales, A. C., R. N. Jones, and H. S. Sader. 2006. Global assessment of the antimicrobial activity of polymyxin B against 54 731 clinical isolates of Gram-

- negative bacilli: report from the SENTRY antimicrobial surveillance programme (2001–2004). *Clinical Microbiology and Infection* 12:315-321.
142. Jian, L. I., Rayner, R. Craig, Nation, L. Roger, Owen, J. Roxanne, Spelman, Denis, T. A. N. Kar Eng, Liolios, and Lisa. 2006. Heteroresistance to colistin in multidrug-resistant *Acinetobacter baumannii*. American Society for Microbiology, Washington, DC, ETATS-UNIS.
 143. Van Looveren, M., H. Goossens, and A. S. G. the. 2004. Antimicrobial resistance of *Acinetobacter* spp. in Europe. *Clinical Microbiology and Infection* 10:684-704.
 144. Lee, K., J. H. Yum, D. Yong, H. M. Lee, H. D. Kim, J.-D. Docquier, G. M. Rossolini, and Y. Chong. 2005. Novel Acquired Metallo- β -Lactamase Gene, blaSIM-1, in a Class 1 Integron from *Acinetobacter baumannii* Clinical Isolates from Korea. *Antimicrob. Agents Chemother.* 49:4485-4491.
 145. Kaper, J. B., J. P. Nataro, and H. L. T. Mobley. 2004. Pathogenic *Escherichia coli*. *Nat Rev Micro* 2:123-140.
 146. Fotadar, U., P. Zaveloff, and L. Terracio. 2005. Growth of *Escherichia coli* at elevated temperatures. *Journal of Basic Microbiology* 45:403-404.
 147. Nataro, J. P., and J. B. Kaper. 1998. Diarrheagenic *Escherichia coli*. *Clin. Microbiol. Rev.* 11:142-201.
 148. Knutton, S., I. Rosenshine, M. J. Pallen, I. Nisan, B. C. Neves, C. Bain, C. Wolff, G. Dougan, and G. Frankel. 1998. A novel EspA-associated surface organelle of enteropathogenic *Escherichia coli* involved in protein translocation into epithelial cells. *EMBO J* 17:2166-2176.

149. Kenny, B., S. Ellis, A. D. Leard, J. Warawa, H. Mellor, and M. A. Jepson. 2002. Co-ordinate regulation of distinct host cell signalling pathways by multifunctional enteropathogenic *Escherichia coli* effector molecules. *Molecular Microbiology* 44:1095-1107.
150. Toshima, H., A. Yoshimura, K. Arikawa, A. Hidaka, J. Ogasawara, A. Hase, H. Masaki, and Y. Nishikawa. 2007. Enhancement of Shiga toxin production in enterohemorrhagic *Escherichia coli* serotype O157:H7 by DNase colicins. *Appl Environ Microbiol* 73:7582-7588.
151. Andreoli, S. P., H. Trachtman, D. W. K. Acheson, R. L. Siegler, and T. G. Obrig. 2002. Hemolytic uremic syndrome: epidemiology, pathophysiology, and therapy. *Pediatric Nephrology* 17:293-298.
152. Sears, L. C, Kaper, and B. J. 1996. Enteric bacterial toxins : Mechanisms of action and linkage to intestinal secretion. American Society for Microbiology, Washington, DC, ETATS-UNIS.
153. Hicks, S., D. C. Candy, and A. D. Phillips. 1996. Adhesion of enteroaggregative *Escherichia coli* to pediatric intestinal mucosa in vitro. *Infect. Immun.* 64:4751-4760.
154. Vial, A. P, B. Robins, R. Lior, H. Prado, V, Kaper, B. J, Nataro, P. J, Maneval, D, Elsayed, A, Levine, and M. M. 1988. Characterization of enteroadherent-aggregative *Escherichia coli*, a putative agent on diarrheal disease. Oxford University Press, Oxford, ROYAUME-UNI.
155. Nataro, J. P., Y. Deng, D. R. Maneval, A. L. German, W. C. Martin, and M. M. Levine. 1992. Aggregative adherence fimbriae I of enteroaggregative *Escherichia*

- coli mediate adherence to HEp-2 cells and hemagglutination of human erythrocytes. *Infect. Immun.* 60:2297-2304.
156. Jiang, Z.-D., D. Greenberg, J. P. Nataro, R. Steffen, and H. L. DuPont. 2002. Rate of Occurrence and Pathogenic Effect of Enteroaggregative *Escherichia coli* Virulence Factors in International Travelers. *J. Clin. Microbiol.* 40:4185-4190.
 157. Zychlinsky, A., M. C. Prevost, and P. J. Sansonetti. 1992. *Shigella flexneri* induces apoptosis in infected macrophages. *Nature* 358:167-169.
 158. Buchrieser, C., P. Glaser, C. Rusniok, H. Nedjari, H. D'Hauteville, F. Kunst, P. Sansonetti, and C. Parsot. 2000. The virulence plasmid pWR100 and the repertoire of proteins secreted by the type III secretion apparatus of *Shigella flexneri*. *Molecular Microbiology* 38:760-771.
 159. Bernet-Camard, M. F., M. H. Coconnier, S. Hudault, and A. L. Servin. 1996. Pathogenicity of the diffusely adhering strain *Escherichia coli* C1845: F1845 adhesin-decay accelerating factor interaction, brush border microvillus injury, and actin disassembly in cultured human intestinal epithelial cells. *Infect. Immun.* 64:1918-1928.
 160. Nowicki, B., C. Svanborg-Eden, R. Hull, and S. Hull. 1989. Molecular analysis and epidemiology of the Dr hemagglutinin of uropathogenic *Escherichia coli*. *Infect. Immun.* 57:446-451.
 161. Johnson, J. R., and A. L. Stell. 2000. Extended Virulence Genotypes of *Escherichia coli* Strains from Patients with Urosepsis in Relation to Phylogeny and Host Compromise. *Journal of Infectious Diseases* 181:261-272.

162. Unhanand, M., M. M. Mustafa, G. H. McCracken, and J. D. Nelson. 1993. Gram-negative enteric bacillary meningitis: A twenty-one-year experience. *The Journal of pediatrics* 122:15-21.
163. Dawson, K. G., J. C. Emerson, and J. L. Burns. 1999. Fifteen years of experience with bacterial meningitis. *The Pediatric infectious disease journal* 18:816-822.
164. Livermore, D. M. 1995. beta-Lactamases in laboratory and clinical resistance. *Clin. Microbiol. Rev.* 8:557-584.
165. Vila, J., J. Ruiz, F. Marco, A. Barcelo, P. Goni, E. Giralt, and T. Jimenez de Anta. 1994. Association between double mutation in *gyrA* gene of ciprofloxacin-resistant clinical isolates of *Escherichia coli* and MICs. *Antimicrob. Agents Chemother.* 38:2477-2479.
166. Wang, H., J. L. Dzink-Fox, M. Chen, and S. B. Levy. 2001. Genetic Characterization of Highly Fluoroquinolone-Resistant Clinical *Escherichia coli* Strains from China: Role of *acrR* Mutations. *Antimicrob. Agents Chemother.* 45:1515-1521.
167. Akoachere, T. K. Jane-Francis, Bughe, N. Rhoda, Oben, O. Benedicta, Ndip, M. Lucy, and N. Roland. 2009. Phenotypic Characterization of Human Pathogenic Bacteria in fish from the Coastal Waters of South West Cameroon: Public Health Implications. Freund, London, ROYAUME-UNI.
168. Jang, S., L. Wheeler, R. B. Carey, B. Jensen, C. M. Crandall, K. N. Schrader, D. Jessup, K. Colegrove, and F. M. D. Gulland. Pleuritis and suppurative pneumonia associated with a hypermucoviscosity phenotype of *Klebsiella*

- pneumoniae in California sea lions (*Zalophus californianus*). *Veterinary Microbiology* 141:174-177.
169. Castinel, A., A. Grinberg, R. Pattison, P. d. Duignan, B. Pomroy, L. Rogers, and I. Wilkinson. 2007. Characterization of *Klebsiella pneumoniae* isolates from New Zealand sea lion (*Phocartos hookeri*) pups during and after the epidemics on Enderby Island, Auckland Islands. *Veterinary Microbiology* 122:178-184.
 170. Podschun, R., and U. Ullmann. 1998. *Klebsiella* spp. as Nosocomial Pathogens: Epidemiology, Taxonomy, Typing Methods, and Pathogenicity Factors. *Clin. Microbiol. Rev.* 11:589-603.
 171. Pollack, M., R. Nieman, J. Reinhardt, P. Charache, M. Jett, and P. Hardy. 1972. FACTORS INFLUENCING COLONISATION AND ANTIBIOTIC-RESISTANCE PATTERNS OF GRAM-NEGATIVE BACTERIA IN HOSPITAL PATIENTS. *The Lancet* 300:668-671.
 172. Niemela, S. I., P. Vaatanen, J. Mentu, A. Jokinen, P. Jappinen, and P. Sillanpaa. 1985. Microbial incidence in upper respiratory tracts of workers in the paper industry. *Appl. Environ. Microbiol.* 50:163-168.
 173. Tsay, R.-W., L. K. Siu, C.-P. Fung, and F.-Y. Chang. 2002. Characteristics of Bacteremia Between Community-Acquired and Nosocomial *Klebsiella pneumoniae* Infection: Risk Factor for Mortality and the Impact of Capsular Serotypes as a Herald for Community-Acquired Infection. *Arch Intern Med* 162:1021-1027.
 174. Lee, H. K, Hui, P. K, Tan, C. W, Lim, and K. T. 1994. KLEBSIELLA BACTERAEMIA : A REPORT OF 101 CASES FROM NATIONAL

UNIVERSITY HOSPITAL, SINGAPORE. Elsevier, Kidlington, ROYAUME-UNI.

175. Feldman, C., C. Smith, H. Levy, P. Ginsburg, S. D. Miller, and H. J. Koornhof. 1990. *Klebsiella pneumoniae* bacteraemia at an urban general hospital. *Journal of Infection* 20:21-31.
176. Watanakunakorn, C, Jura, and J. 1991. *Klebsiella* bacteremia : a review of 196 episodes during a decade (1980-1989). Informa, Colchester, ROYAUME-UNI.
177. Pitout, J. D. D., K. S. Thomson, N. D. Hanson, A. F. Ehrhardt, E. S. Moland, and C. C. Sanders. 1998. beta -Lactamases Responsible for Resistance to Expanded-Spectrum Cephalosporins in *Klebsiella pneumoniae*, *Escherichia coli*, and *Proteus mirabilis* Isolates Recovered in South Africa. *Antimicrob. Agents Chemother.* 42:1350-1354.
178. Lee, C.-H., C. Chu, J.-W. Liu, Y.-S. Chen, C.-J. Chiu, and L.-H. Su. 2007. Collateral damage of flomoxef therapy: in vivo development of porin deficiency and acquisition of blaDHA-1 leading to ertapenem resistance in a clinical isolate of *Klebsiella pneumoniae* producing CTX-M-3 and SHV-5 {beta}-lactamases. *Journal of Antimicrobial Chemotherapy* 60:410-413.
179. Cryz, S. J., Jr., P. M. Mortimer, V. Mansfield, and R. Germanier. 1986. Seroepidemiology of *Klebsiella* bacteremic isolates and implications for vaccine development. *J. Clin. Microbiol.* 23:687-690.
180. Fung, C.-P., B.-S. Hu, F.-Y. Chang, S.-C. Lee, B. In-Tiau Kuo, M. Ho, L. K. Siu, and C.-Y. Liu. 2000. A 5-Year Study of the Seroepidemiology of *Klebsiella*

- pneumoniae: High Prevalence of Capsular Serotype K1 in Taiwan and Implication for Vaccine Efficacy. *Journal of Infectious Diseases* 181:2075-2079.
181. Yeh, K.-M., A. Kurup, L. K. Siu, Y. L. Koh, C.-P. Fung, J.-C. Lin, T.-L. Chen, F.-Y. Chang, and T.-H. Koh. 2007. Capsular Serotype K1 or K2, Rather than *magA* and *rpmA*, Is a Major Virulence Determinant for *Klebsiella pneumoniae* Liver Abscess in Singapore and Taiwan. *J. Clin. Microbiol.* 45:466-471.
 182. Fang, C.-T., S.-Y. Lai, W.-C. Yi, P.-R. Hsueh, K.-L. Liu, and S.-C. Chang. 2007. *Klebsiella pneumoniae* Genotype K1: An Emerging Pathogen That Causes Septic Ocular or Central Nervous System Complications from Pyogenic Liver Abscess. *Clinical Infectious Diseases* 45:284-293.
 183. Jenney, A. W., A. Clements, J. L. Farn, O. L. Wijburg, A. McGlinchey, D. W. Spelman, T. L. Pitt, M. E. Kaufmann, L. Liolios, M. B. Moloney, S. L. Wesselingh, and R. A. Strugnell. 2006. Seroepidemiology of *Klebsiella pneumoniae* in an Australian Tertiary Hospital and Its Implications for Vaccine Development. *J. Clin. Microbiol.* 44:102-107.
 184. Blanchette, E. A., and S. J. Rubin. 1980. Seroepidemiology of clinical isolates of *Klebsiella* in Connecticut. *J. Clin. Microbiol.* 11:474-478.
 185. Mizuta, K., M. Ohta, M. Mori, T. Hasegawa, I. Nakashima, and N. Kato. 1983. Virulence for mice of *Klebsiella* strains belonging to the O1 group: relationship to their capsular (K) types. *Infect. Immun.* 40:56-61.
 186. Lin, J.-C., F.-Y. Chang, C.-P. Fung, J.-Z. Xu, H.-P. Cheng, J.-J. Wang, L.-Y. Huang, and L. K. Siu. 2004. High prevalence of phagocytic-resistant capsular

- serotypes of *Klebsiella pneumoniae* in liver abscess. *Microbes and Infection* 6:1191-1198.
187. Fang, T. Chi, Chuang, P. Yi, Shun, T. Chia, Chang, C. Shan, Wang, and T. Jin. 2004. A novel virulence gene in *Klebsiella pneumoniae* strains causing primary liver abscess and septic metastatic complications. Rockefeller University Press, New York, NY, ETATS-UNIS.
 188. Alvarez, D., S. Merino, J. M. Tomas, V. J. Benedi, and S. Alberti. 2000. Capsular Polysaccharide Is a Major Complement Resistance Factor in Lipopolysaccharide O Side Chain-Deficient *Klebsiella pneumoniae* Clinical Isolates. *Infect. Immun.* 68:953-955.
 189. Yu, W.-L., W.-C. Ko, K.-C. Cheng, H.-C. Lee, D.-S. Ke, C.-C. Lee, C.-P. Fung, and Y.-C. Chuang. 2006. Association between *rmpA* and *magA* Genes and Clinical Syndromes Caused by *Klebsiella pneumoniae* in Taiwan. *Clinical Infectious Diseases* 42:1351-1358.
 190. Lee, H. C., Y. C. Chuang, W. L. Yu, N. Y. Lee, C. M. Chang, N. Y. Ko, L. R. Wang, and W. C. Ko. 2006. Clinical implications of hypermucoviscosity phenotype in *Klebsiella pneumoniae* isolates: association with invasive syndrome in patients with community-acquired bacteraemia. *Journal of Internal Medicine* 259:606-614.
 191. Nadasy, K. A., R. Domiati-Saad, and M. A. Tribble. 2007. Invasive *Klebsiella pneumoniae* Syndrome in North America. *Clinical Infectious Diseases* 45:e25-e28.

192. Tomas, J. M., V. J. Benedi, B. Ciurana, and J. Jofre. 1986. Role of capsule and O antigen in resistance of *Klebsiella pneumoniae* to serum bactericidal activity. *Infect. Immun.* 54:85-89.
193. McCallum, K. L., G. Schoenhals, D. Laakso, B. Clarke, and C. Whitfield. 1989. A high-molecular-weight fraction of smooth lipopolysaccharide in *Klebsiella* serotype O1:K20 contains a unique O-antigen epitope and determines resistance to nonspecific serum killing. *Infect. Immun.* 57:3816-3822.
194. Shankar-Sinha, S., G. A. Valencia, B. K. Janes, J. K. Rosenberg, C. Whitfield, R. A. Bender, T. J. Standiford, and J. G. Younger. 2004. The *Klebsiella pneumoniae* O Antigen Contributes to Bacteremia and Lethality during Murine Pneumonia. *Infect. Immun.* 72:1423-1430.
195. Miles, A. A., and P. L. Khimji. 1975. ENTEROBACTERIAL CHELATORS OF IRON: THEIR OCCURRENCE, DETECTION, AND RELATION TO PATHOGENICITY. *J Med Microbiol* 8:477-490.
196. Podschun, R, Fischer, A, Ullmann, and U. 1992. Siderophore production of *Klebsiella* species isolated from different sources. Fischer, Jena, ALLEMAGNE.
197. Podschun, R., D. Sievers, A. Fischer, and U. Ullmann. 1993. Serotypes, Hemagglutinins, Siderophore Synthesis, and Serum Resistance of *Klebsiella* Isolates Causing Human Urinary Tract Infections. *Journal of Infectious Diseases* 168:1415-1421.
198. Tarkkanen, A. M., B. L. Allen, P. H. Williams, M. Kauppi, K. Haahtela, A. Siitonen, I. Orskov, F. Orskov, S. Clegg, and T. K. Korhonen. 1992. Fimbriation,

- capsulation, and iron-scavenging systems of *Klebsiella* strains associated with human urinary tract infection. *Infect. Immun.* 60:1187-1192.
199. Nassif, X., and P. J. Sansonetti. 1986. Correlation of the virulence of *Klebsiella pneumoniae* K1 and K2 with the presence of a plasmid encoding aerobactin. *Infect Immun* 54:603-608.
 200. Gerlach, G. F., S. Clegg, and B. L. Allen. 1989. Identification and characterization of the genes encoding the type 3 and type 1 fimbrial adhesins of *Klebsiella pneumoniae*. *J Bacteriol* 171:1262-1270.
 201. Jones, C. H., J. S. Pinkner, R. Roth, J. Heuser, A. V. Nicholes, S. N. Abraham, and S. J. Hultgren. 1995. FimH adhesin of type 1 pili is assembled into a fibrillar tip structure in the Enterobacteriaceae. *Proc Natl Acad Sci U S A* 92:2081-2085.
 202. Yu, W. L., W. C. Ko, K. C. Cheng, H. C. Lee, D. S. Ke, C. C. Lee, C. P. Fung, and Y. C. Chuang. 2006. Association between *rmpA* and *magA* genes and clinical syndromes caused by *Klebsiella pneumoniae* in Taiwan. *Clin Infect Dis* 42:1351-1358.
 203. Sebghati, T. A., T. K. Korhonen, D. B. Hornick, and S. Clegg. 1998. Characterization of the type 3 fimbrial adhesins of *Klebsiella* strains. *Infect Immun* 66:2887-2894.
 204. Jagnow, J., and S. Clegg. 2003. *Klebsiella pneumoniae* MrkD-mediated biofilm formation on extracellular matrix- and collagen-coated surfaces. *Microbiology* 149:2397-2405.
 205. Aslangul, E., L. Massias, A. Meulemans, F. Chau, A. Andremont, P. Courvalin, B. Fantin, and R. Ruimy. 2006. Acquired gentamicin resistance by permeability

- impairment in *Enterococcus faecalis*. *Antimicrob Agents Chemother* 50:3615-3621.
206. Courvalin, P. 2006. Vancomycin resistance in gram-positive cocci. *Clin Infect Dis* 42 Suppl 1:S25-34.
 207. Marothi, Y. A., H. Agnihotri, and D. Dubey. 2005. Enterococcal resistance--an overview. *Indian J Med Microbiol* 23:214-219.
 208. Huycke, M. M., D. F. Sahm, and M. S. Gilmore. 1998. Multiple-drug resistant enterococci: the nature of the problem and an agenda for the future. *Emerg Infect Dis* 4:239-249.
 209. Olmsted, S. B., S. M. Kao, L. J. van Putte, J. C. Gallo, and G. M. Dunny. 1991. Role of the pheromone-inducible surface protein Asc10 in mating aggregate formation and conjugal transfer of the *Enterococcus faecalis* plasmid pCF10. *J Bacteriol* 173:7665-7672.
 210. Dunny, G. M., B. A. Leonard, and P. J. Hedberg. 1995. Pheromone-inducible conjugation in *Enterococcus faecalis*: interbacterial and host-parasite chemical communication. *J Bacteriol* 177:871-876.
 211. Hedberg, P. J., B. A. Leonard, R. E. Ruhfel, and G. M. Dunny. 1996. Identification and characterization of the genes of *Enterococcus faecalis* plasmid pCF10 involved in replication and in negative control of pheromone-inducible conjugation. *Plasmid* 35:46-57.
 212. Waters, C. M., M. H. Antiporta, B. E. Murray, and G. M. Dunny. 2003. Role of the *Enterococcus faecalis* GelE protease in determination of cellular chain length,

- supernatant pheromone levels, and degradation of fibrin and misfolded surface proteins. *J Bacteriol* 185:3613-3623.
213. Sussmuth, S. D., A. Muscholl-Silberhorn, R. Wirth, M. Susa, R. Marre, and E. Rozdzinski. 2000. Aggregation substance promotes adherence, phagocytosis, and intracellular survival of *Enterococcus faecalis* within human macrophages and suppresses respiratory burst. *Infect Immun* 68:4900-4906.
 214. Wells, C. L., E. A. Moore, J. A. Hoag, H. Hirt, G. M. Dunny, and S. L. Erlandsen. 2000. Inducible expression of *Enterococcus faecalis* aggregation substance surface protein facilitates bacterial internalization by cultured enterocytes. *Infect Immun* 68:7190-7194.
 215. Archimbaud, C., N. Shankar, C. Forestier, A. Baghdayan, M. S. Gilmore, F. Charbonne, and B. Joly. 2002. In vitro adhesive properties and virulence factors of *Enterococcus faecalis* strains. *Res Microbiol* 153:75-80.
 216. Leavis, H., J. Top, N. Shankar, K. Borgen, M. Bonten, J. van Embden, and R. J. Willems. 2004. A novel putative enterococcal pathogenicity island linked to the *esp* virulence gene of *Enterococcus faecium* and associated with epidemicity. *J Bacteriol* 186:672-682.
 217. Shankar, N., C. V. Lockatell, A. S. Baghdayan, C. Drachenberg, M. S. Gilmore, and D. E. Johnson. 2001. Role of *Enterococcus faecalis* surface protein Esp in the pathogenesis of ascending urinary tract infection. *Infect Immun* 69:4366-4372.
 218. Hancock, L. E., and M. Perego. 2004. The *Enterococcus faecalis* *fsr* two-component system controls biofilm development through production of gelatinase. *J Bacteriol* 186:5629-5639.

219. Nallapareddy, S. R., K. V. Singh, J. Sillanpaa, D. A. Garsin, M. Hook, S. L. Erlandsen, and B. E. Murray. 2006. Endocarditis and biofilm-associated pili of *Enterococcus faecalis*. *J Clin Invest* 116:2799-2807.
220. Kemp, K. D., K. V. Singh, S. R. Nallapareddy, and B. E. Murray. 2007. Relative contributions of *Enterococcus faecalis* OG1RF sortase-encoding genes, *srtA* and *bps* (*srtC*), to biofilm formation and a murine model of urinary tract infection. *Infect Immun* 75:5399-5404.
221. Singh, K. V., S. R. Nallapareddy, and B. E. Murray. 2007. Importance of the *ebp* (endocarditis- and biofilm-associated pilus) locus in the pathogenesis of *Enterococcus faecalis* ascending urinary tract infection. *J Infect Dis* 195:1671-1677.
222. Thurlow, L. R., V. C. Thomas, and L. E. Hancock. 2009. Capsular polysaccharide production in *Enterococcus faecalis* and contribution of *CpsF* to capsule serospecificity. *J Bacteriol* 191:6203-6210.
223. Sava, I. G., F. Zhang, I. Toma, C. Theilacker, B. Li, T. F. Baumert, O. Holst, R. J. Linhardt, and J. Huebner. 2009. Novel interactions of glycosaminoglycans and bacterial glycolipids mediate binding of enterococci to human cells. *J Biol Chem* 284:18194-18201.
224. Theilacker, C., P. Sanchez-Carballo, I. Toma, F. Fabretti, I. Sava, A. Kropec, O. Holst, and J. Huebner. 2009. Glycolipids are involved in biofilm accumulation and prolonged bacteraemia in *Enterococcus faecalis*. *Mol Microbiol* 71:1055-1069.

225. Murray, B. E. 1990. The life and times of the Enterococcus. Clin Microbiol Rev 3:46-65.
226. Rhinehart, E., N. E. Smith, C. Wennersten, E. Gorss, J. Freeman, G. M. Eliopoulos, R. C. Moellering, Jr., and D. A. Goldmann. 1990. Rapid dissemination of beta-lactamase-producing, aminoglycoside-resistant Enterococcus faecalis among patients and staff on an infant-toddler surgical ward. N Engl J Med 323:1814-1818.
227. Rice, L. B. 2005. Antibiotics and gastrointestinal colonization by vancomycin-resistant enterococci. Eur J Clin Microbiol Infect Dis 24:804-814.
228. Wells, V. D., E. S. Wong, B. E. Murray, P. E. Coudron, D. S. Williams, and S. M. Markowitz. 1992. Infections due to beta-lactamase-producing, high-level gentamicin-resistant Enterococcus faecalis. Ann Intern Med 116:285-292.
229. Morris, J. G., Jr., D. K. Shay, J. N. Hebden, R. J. McCarter, Jr., B. E. Perdue, W. Jarvis, J. A. Johnson, T. C. Dowling, L. B. Polish, and R. S. Schwalbe. 1995. Enterococci resistant to multiple antimicrobial agents, including vancomycin. Establishment of endemicity in a university medical center. Ann Intern Med 123:250-259.
230. Bhat, K. G., C. Paul, and M. G. Bhat. 1997. High level aminoglycoside resistance in enterococci isolated from hospitalized patients. Indian J Med Res 105:198-199.
231. Leclercq, R., E. Derlot, J. Duval, and P. Courvalin. 1988. Plasmid-mediated resistance to vancomycin and teicoplanin in Enterococcus faecium. N Engl J Med 319:157-161.

232. Montecalvo, M. A., D. K. Shay, P. Patel, L. Tacsá, S. A. Maloney, W. R. Jarvis, and G. P. Wormser. 1996. Bloodstream infections with vancomycin-resistant enterococci. *Arch Intern Med* 156:1458-1462.
233. Uttley, A. H., C. H. Collins, J. Naidoo, and R. C. George. 1988. Vancomycin-resistant enterococci. *Lancet* 1:57-58.
234. Leclercq, R., S. Dutka-Malen, A. Brisson-Noel, C. Molinas, E. Derlot, M. Arthur, J. Duval, and P. Courvalin. 1992. Resistance of enterococci to aminoglycosides and glycopeptides. *Clin Infect Dis* 15:495-501.
235. Leclercq, R., S. Dutka-Malen, J. Duval, and P. Courvalin. 1992. Vancomycin resistance gene vanC is specific to *Enterococcus gallinarum*. *Antimicrob Agents Chemother* 36:2005-2008.
236. Lowy, F. D. 1998. *Staphylococcus aureus* infections. *N Engl J Med* 339:520-532.
237. Farley, J. E. 2008. Epidemiology, clinical manifestations, and treatment options for skin and soft tissue infection caused by community-acquired methicillin-resistant *Staphylococcus aureus*. *J Am Acad Nurse Pract* 20:85-92.
238. Farley, J. E., T. Ross, P. Stamper, S. Baucom, E. Larson, and K. C. Carroll. 2008. Prevalence, risk factors, and molecular epidemiology of methicillin-resistant *Staphylococcus aureus* among newly arrested men in Baltimore, Maryland. *Am J Infect Control* 36:644-650.
239. Gonzalez, B. E., G. Martinez-Aguilar, K. G. Hulten, W. A. Hammerman, J. Coss-Bu, A. Avalos-Mishaan, E. O. Mason, Jr., and S. L. Kaplan. 2005. Severe *Staphylococcal* sepsis in adolescents in the era of community-acquired methicillin-resistant *Staphylococcus aureus*. *Pediatrics* 115:642-648.

240. Guidet, B., P. Aegerter, R. Gauzit, P. Meshaka, and D. Dreyfuss. 2005. Incidence and impact of organ dysfunctions associated with sepsis. *Chest* 127:942-951.
241. Balaban, N., and A. Rasooly. 2000. Staphylococcal enterotoxins. *Int J Food Microbiol* 61:1-10.
242. Athanassa, Z., Siempos, II, and M. E. Falagas. 2008. Impact of methicillin resistance on mortality in *Staphylococcus aureus* VAP: a systematic review. *Eur Respir J* 31:625-632.
243. Plata, K., A. E. Rosato, and G. Wegrzyn. 2009. *Staphylococcus aureus* as an infectious agent: overview of biochemistry and molecular genetics of its pathogenicity. *Acta Biochim Pol* 56:597-612.
244. Smeltzer, M. S., and A. F. Gillaspay. 2000. Molecular pathogenesis of staphylococcal osteomyelitis. *Poult Sci* 79:1042-1049.
245. Madaras-Kelly, K. J., R. E. Remington, C. M. Oliphant, K. L. Sloan, and D. T. Bearden. 2008. Efficacy of oral beta-lactam versus non-beta-lactam treatment of uncomplicated cellulitis. *Am J Med* 121:419-425.
246. Herold, B. C., L. C. Immergluck, M. C. Maranan, D. S. Lauderdale, R. E. Gaskin, S. Boyle-Vavra, C. D. Leitch, and R. S. Daum. 1998. Community-acquired methicillin-resistant *Staphylococcus aureus* in children with no identified predisposing risk. *JAMA* 279:593-598.
247. Gorak, E. J., S. M. Yamada, and J. D. Brown. 1999. Community-acquired methicillin-resistant *Staphylococcus aureus* in hospitalized adults and children without known risk factors. *Clin Infect Dis* 29:797-800.

248. Diep, B. A., H. F. Chambers, C. J. Graber, J. D. Szumowski, L. G. Miller, L. L. Han, J. H. Chen, F. Lin, J. Lin, T. H. Phan, H. A. Carleton, L. K. McDougal, F. C. Tenover, D. E. Cohen, K. H. Mayer, G. F. Sensabaugh, and F. Perdreau-Remington. 2008. Emergence of multidrug-resistant, community-associated, methicillin-resistant *Staphylococcus aureus* clone USA300 in men who have sex with men. *Ann Intern Med* 148:249-257.
249. Diep, B. A., S. R. Gill, R. F. Chang, T. H. Phan, J. H. Chen, M. G. Davidson, F. Lin, J. Lin, H. A. Carleton, E. F. Mongodin, G. F. Sensabaugh, and F. Perdreau-Remington. 2006. Complete genome sequence of USA300, an epidemic clone of community-acquired methicillin-resistant *Staphylococcus aureus*. *Lancet* 367:731-739.
250. Han, L. L., L. K. McDougal, R. J. Gorwitz, K. H. Mayer, J. B. Patel, J. M. Sennott, and J. L. Fontana. 2007. High frequencies of clindamycin and tetracycline resistance in methicillin-resistant *Staphylococcus aureus* pulsed-field type USA300 isolates collected at a Boston ambulatory health center. *J Clin Microbiol* 45:1350-1352.
251. Mishra, N. N., S. J. Yang, A. Sawa, A. Rubio, C. C. Nast, M. R. Yeaman, and A. S. Bayer. 2009. Analysis of cell membrane characteristics of in vitro-selected daptomycin-resistant strains of methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother* 53:2312-2318.
252. Cole, A. M., P. Weis, and G. Diamond. 1997. Isolation and characterization of pleurocidin, an antimicrobial peptide in the skin secretions of winter flounder. *J Biol Chem* 272:12008-12013.

- 253. Cole, S. R., and J. G. Dorsey. 1997. Cyclohexylamine additives for enhanced peptide separations in reversed phase liquid chromatography. *Biomed Chromatogr* 11:167-171.
- 254. Hancock, R. E., and G. Diamond. 2000. The role of cationic antimicrobial peptides in innate host defences. *Trends Microbiol* 8:402-410.
- 255. Murphy, C. J., B. A. Foster, M. J. Mannis, M. E. Selsted, and T. W. Reid. 1993. Defensins are mitogenic for epithelial cells and fibroblasts. *J Cell Physiol* 155:408-413.
- 256. Territo, M. C., T. Ganz, M. E. Selsted, and R. Lehrer. 1989. Monocyte-chemotactic activity of defensins from human neutrophils. *J Clin Invest* 84:2017-2020.
- 257. Caverly, J. M., G. Diamond, J. M. Gallup, K. A. Brogden, R. A. Dixon, and M. R. Ackermann. 2003. Coordinated expression of tracheal antimicrobial peptide and inflammatory-response elements in the lungs of neonatal calves with acute bacterial pneumonia. *Infect Immun* 71:2950-2955.
- 258. Zasloff, M. 2002. Antimicrobial peptides of multicellular organisms. *Nature* 415:389-395.
- 259. Jones, M. K., G. M. Anantharamaiah, and J. P. Segrest. 1992. Computer programs to identify and classify amphipathic alpha helical domains. *J Lipid Res* 33:287-296.
- 260. Javadpour, M. M., and M. D. Barkley. 1997. Self-assembly of designed antimicrobial peptides in solution and micelles. *Biochemistry* 36:9540-9549.

- 261. Brogden, K. A., M. Ackermann, and K. M. Huttner. 1998. Detection of anionic antimicrobial peptides in ovine bronchoalveolar lavage fluid and respiratory epithelium. *Infect Immun* 66:5948-5954.
- 262. Gennaro, R., and M. Zanetti. 2000. Structural features and biological activities of the cathelicidin-derived antimicrobial peptides. *Biopolymers* 55:31-49.
- 263. Hancock, R. E. 1997. Peptide antibiotics. *Lancet* 349:418-422.
- 264. Brogden, K. A., A. J. De Lucca, J. Bland, and S. Elliott. 1996. Isolation of an ovine pulmonary surfactant-associated anionic peptide bactericidal for *Pasteurella haemolytica*. *Proc Natl Acad Sci U S A* 93:412-416.
- 265. Brogden, K. A., M. R. Ackermann, P. B. McCray, Jr., and K. M. Huttner. 1999. Differences in the concentrations of small, anionic, antimicrobial peptides in bronchoalveolar lavage fluid and in respiratory epithelia of patients with and without cystic fibrosis. *Infect Immun* 67:4256-4259.
- 266. Zanetti, M., R. Gennaro, M. Scocchi, and B. Skerlavaj. 2000. Structure and biology of cathelicidins. *Adv Exp Med Biol* 479:203-218.
- 267. Tossi, A., L. Sandri, and A. Giangaspero. 2000. Amphipathic, alpha-helical antimicrobial peptides. *Biopolymers* 55:4-30.
- 268. Brogden, K. A. 2005. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat Rev Microbiol* 3:238-250.
- 269. Pouny, Y., D. Rapaport, A. Mor, P. Nicolas, and Y. Shai. 1992. Interaction of antimicrobial dermaseptin and its fluorescently labeled analogues with phospholipid membranes. *Biochemistry* 31:12416-12423.

270. Shai, Y. 1999. Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by alpha-helical antimicrobial and cell non-selective membrane-lytic peptides. *Biochim Biophys Acta* 1462:55-70.
271. Ladokhin, A. S., and S. H. White. 2001. 'Detergent-like' permeabilization of anionic lipid vesicles by melittin. *Biochim Biophys Acta* 1514:253-260.
272. Matsuzaki, K., O. Murase, N. Fujii, and K. Miyajima. 1996. An antimicrobial peptide, magainin 2, induced rapid flip-flop of phospholipids coupled with pore formation and peptide translocation. *Biochemistry* 35:11361-11368.
273. Dathe, M., and T. Wieprecht. 1999. Structural features of helical antimicrobial peptides: their potential to modulate activity on model membranes and biological cells. *Biochim Biophys Acta* 1462:71-87.
274. Easton, D. M., A. Nijnik, M. L. Mayer, and R. E. Hancock. 2009. Potential of immunomodulatory host defense peptides as novel anti-infectives. *Trends Biotechnol* 27:582-590.
275. Marr, A. K., W. J. Gooderham, and R. E. Hancock. 2006. Antibacterial peptides for therapeutic use: obstacles and realistic outlook. *Curr Opin Pharmacol* 6:468-472.
276. Silverman, J. A., N. G. Perlmutter, and H. M. Shapiro. 2003. Correlation of daptomycin bactericidal activity and membrane depolarization in *Staphylococcus aureus*. *Antimicrob Agents Chemother* 47:2538-2544.
277. Barry, A. L., P. C. Fuchs, and S. D. Brown. 2001. In vitro activities of daptomycin against 2,789 clinical isolates from 11 North American medical centers. *Antimicrob Agents Chemother* 45:1919-1922.

278. Critchley, I. A., R. S. Blosser-Middleton, M. E. Jones, C. Thornsberry, D. F. Sahm, and J. A. Karlowsky. 2003. Baseline study to determine in vitro activities of daptomycin against gram-positive pathogens isolated in the United States in 2000-2001. *Antimicrob Agents Chemother* 47:1689-1693.
279. Rybak, M. J., E. Hershberger, T. Moldovan, and R. G. Grucz. 2000. In vitro activities of daptomycin, vancomycin, linezolid, and quinupristin-dalfopristin against *Staphylococci* and *Enterococci*, including vancomycin- intermediate and -resistant strains. *Antimicrob Agents Chemother* 44:1062-1066.
280. Snyderman, D. R., N. V. Jacobus, L. A. McDermott, J. R. Lonks, and J. M. Boyce. 2000. Comparative In vitro activities of daptomycin and vancomycin against resistant gram-positive pathogens. *Antimicrob Agents Chemother* 44:3447-3450.
281. Streit, J. M., R. N. Jones, and H. S. Sader. 2004. Daptomycin activity and spectrum: a worldwide sample of 6737 clinical Gram-positive organisms. *J Antimicrob Chemother* 53:669-674.
282. Canepari, P., M. Boaretti, M. M. Lleo, and G. Satta. 1990. Lipoteichoic acid as a new target for activity of antibiotics: mode of action of daptomycin (LY146032). *Antimicrob Agents Chemother* 34:1220-1226.
283. Dathe, M., T. Wieprecht, H. Nikolenko, L. Handel, W. L. Maloy, D. L. MacDonald, M. Beyermann, and M. Bienert. 1997. Hydrophobicity, hydrophobic moment and angle subtended by charged residues modulate antibacterial and haemolytic activity of amphipathic helical peptides. *FEBS Lett* 403:208-212.

284. Kerth, A., A. Erbe, M. Dathe, and A. Blume. 2004. Infrared reflection absorption spectroscopy of amphipathic model peptides at the air/water interface. *Biophys J* 86:3750-3758.
285. Krause, E., M. Beyermann, M. Dathe, S. Rothemund, and M. Bienert. 1995. Location of an amphipathic alpha-helix in peptides using reversed-phase HPLC retention behavior of D-amino acid analogs. *Anal Chem* 67:252-258.
286. Rothemund, S., E. Krause, M. Beyermann, M. Dathe, H. Engelhardt, and M. Bienert. 1995. Recognition of alpha-helical peptide structures using high-performance liquid chromatographic retention data for D-amino acid analogues: influence of peptide amphipathicity and of stationary phase hydrophobicity. *J Chromatogr A* 689:219-226.
287. Wieprecht, T., S. Rothemund, M. Bienert, and E. Krause. 2001. Role of helix formation for the retention of peptides in reversed-phase high-performance liquid chromatography. *J Chromatogr A* 912:1-12.
288. Wieprecht, T., M. Beyermann, and J. Seelig. 2002. Thermodynamics of the coil-alpha-helix transition of amphipathic peptides in a membrane environment: the role of vesicle curvature. *Biophys Chem* 96:191-201.
289. Javadpour, M. M., M. M. Juban, W. C. Lo, S. M. Bishop, J. B. Alberty, S. M. Cowell, C. L. Becker, and M. L. McLaughlin. 1996. De novo antimicrobial peptides with low mammalian cell toxicity. *J Med Chem* 39:3107-3113.
290. Lee, S., T. Kanmera, H. Aoyagi, and N. Izumiya. 1979. Cyclic peptides. VI. Asymmetric hydrogenation of dehydroalanine or dehydroaminobutanoic acid residue in cyclodipeptides. *Int J Pept Protein Res* 13:207-217.

291. Davies, S. M., T. A. Harroun, T. Hauss, S. M. Kelly, and J. P. Bradshaw. 2003. The membrane bound N-terminal domain of human adenosine diphosphate ribosylation factor-1 (ARF1). *FEBS Lett* 548:119-124.
292. Lee, H. S., F. A. Syud, X. Wang, and S. H. Gellman. 2001. Diversity in short beta-peptide 12-helices: high-resolution structural analysis in aqueous solution of a hexamer containing sulfonylated pyrrolidine residues. *J Am Chem Soc* 123:7721-7722.
293. Wade, D., A. Boman, B. Wahlin, C. M. Drain, D. Andreu, H. G. Boman, and R. B. Merrifield. 1990. All-D amino acid-containing channel-forming antibiotic peptides. *Proc Natl Acad Sci U S A* 87:4761-4765.
294. Bland, J. M., A. J. De Lucca, T. J. Jacks, and C. B. Vigo. 2001. All-D-cecropin B: synthesis, conformation, lipopolysaccharide binding, and antibacterial activity. *Mol Cell Biochem* 218:105-111.
295. Elmquist, A., and U. Langel. 2003. In vitro uptake and stability study of pVEC and its all-D analog. *Biol Chem* 384:387-393.
296. Vunnam, S., P. Juvvadi, K. S. Rotondi, and R. B. Merrifield. 1998. Synthesis and study of normal, enantio, retro, and retroenantio isomers of cecropin A-melittin hybrids, their end group effects and selective enzyme inactivation. *J Pept Res* 51:38-44.
297. Chen, Y., A. I. Vasil, L. Rehaume, C. T. Mant, J. L. Burns, M. L. Vasil, R. E. Hancock, and R. S. Hodges. 2006. Comparison of biophysical and biologic properties of alpha-helical enantiomeric antimicrobial peptides. *Chem Biol Drug Des* 67:162-173.

298. Thomson, C. J., E. Power, H. Ruebsamen-Waigmann, and H. Labischinski. 2004. Antibacterial research and development in the 21(st) Century--an industry perspective of the challenges. *Curr Opin Microbiol* 7:445-450.
299. Poole, K. 2003. Overcoming multidrug resistance in gram-negative bacteria. *Curr Opin Investig Drugs* 4:128-139.
300. Jones, R. N., C. H. Ballow, and D. J. Biedenbach. 2001. Multi-laboratory assessment of the linezolid spectrum of activity using the Kirby-Bauer disk diffusion method: Report of the Zyvox Antimicrobial Potency Study (ZAPS) in the United States. *Diagn Microbiol Infect Dis* 40:59-66.
301. Hayden, M. K., K. Rezai, R. A. Hayes, K. Lolans, J. P. Quinn, and R. A. Weinstein. 2005. Development of Daptomycin resistance in vivo in methicillin-resistant *Staphylococcus aureus*. *J Clin Microbiol* 43:5285-5287.
302. Botelho, M. G. 2000. Fractional inhibitory concentration index of combinations of antibacterial agents against cariogenic organisms. *J Dent* 28:565-570.
303. Merritt, J. H., D. E. Kadouri, and G. A. O'Toole. 2005. Growing and analyzing static biofilms. *Curr Protoc Microbiol* Chapter 1:Unit 1B 1.
304. Mukhopadhyay, K., W. Whitmire, Y. Q. Xiong, J. Molden, T. Jones, A. Peschel, P. Staubitz, J. Adler-Moore, P. J. McNamara, R. A. Proctor, M. R. Yeaman, and A. S. Bayer. 2007. In vitro susceptibility of *Staphylococcus aureus* to thrombin-induced platelet microbicidal protein-1 (tPMP-1) is influenced by cell membrane phospholipid composition and asymmetry. *Microbiology* 153:1187-1197.
305. Gerlag, D. M., E. Borges, P. P. Tak, H. M. Ellerby, D. E. Bredesen, R. Pasqualini, E. Ruoslahti, and G. S. Firestein. 2001. Suppression of murine

- collagen-induced arthritis by targeted apoptosis of synovial neovasculature. *Arthritis Res* 3:357-361.
306. Mai, J. C., Z. Mi, S. H. Kim, B. Ng, and P. D. Robbins. 2001. A proapoptotic peptide for the treatment of solid tumors. *Cancer Res* 61:7709-7712.
 307. Kwon, M. K., J. O. Nam, R. W. Park, B. H. Lee, J. Y. Park, Y. R. Byun, S. Y. Kim, I. C. Kwon, and I. S. Kim. 2008. Antitumor effect of a transducible fusogenic peptide releasing multiple proapoptotic peptides by caspase-3. *Mol Cancer Ther* 7:1514-1522.
 308. Ko, Y. T., C. Falcao, and V. P. Torchilin. 2009. Cationic liposomes loaded with proapoptotic peptide D-(KLAKLAK)(2) and Bcl-2 antisense oligodeoxynucleotide G3139 for enhanced anticancer therapy. *Mol Pharm* 6:971-977.
 309. Ellerby, H. M., W. Arap, L. M. Ellerby, R. Kain, R. Andrusiak, G. D. Rio, S. Krajewski, C. R. Lombardo, R. Rao, E. Ruoslahti, D. E. Bredesen, and R. Pasqualini. 1999. Anti-cancer activity of targeted pro-apoptotic peptides. *Nat Med* 5:1032-1038.
 310. Wheat, P. F. 2001. History and development of antimicrobial susceptibility testing methodology. *J Antimicrob Chemother* 48 Suppl 1:1-4.
 311. Patel, S. 2007. Managing MRSA in hospital and in the community. *Nurs Times* 103:48-49.
 312. DiazGranados, C. A., S. M. Zimmer, M. Klein, and J. A. Jernigan. 2005. Comparison of mortality associated with vancomycin-resistant and vancomycin-

- susceptible enterococcal bloodstream infections: a meta-analysis. *Clin Infect Dis* 41:327-333.
313. Boucher, H. W., G. H. Talbot, J. S. Bradley, J. E. Edwards, D. Gilbert, L. B. Rice, M. Scheld, B. Spellberg, and J. Bartlett. 2009. Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. *Clin Infect Dis* 48:1-12.
 314. Canton, R., P. Ruiz-Garbajosa, R. L. Chaves, and A. P. Johnson. 2010. A potential role for daptomycin in enterococcal infections: what is the evidence? *J Antimicrob Chemother* 65:1126-1136.
 315. Price, J. T., J. M. Quinn, N. A. Sims, J. Vieusseux, K. Waldeck, S. E. Docherty, D. Myers, A. Nakamura, M. C. Waltham, M. T. Gillespie, and E. W. Thompson. 2005. The heat shock protein 90 inhibitor, 17-allylamino-17-demethoxygeldanamycin, enhances osteoclast formation and potentiates bone metastasis of a human breast cancer cell line. *Cancer Res* 65:4929-4938.
 316. Lewis, J. S., 2nd, A. Owens, J. Cadena, K. Sabol, J. E. Patterson, and J. H. Jorgensen. 2005. Emergence of daptomycin resistance in *Enterococcus faecium* during daptomycin therapy. *Antimicrob Agents Chemother* 49:1664-1665.
 317. Jones, T., M. R. Yeaman, G. Sakoulas, S. J. Yang, R. A. Proctor, H. G. Sahl, J. Schrenzel, Y. Q. Xiong, and A. S. Bayer. 2008. Failures in clinical treatment of *Staphylococcus aureus* Infection with daptomycin are associated with alterations in surface charge, membrane phospholipid asymmetry, and drug binding. *Antimicrob Agents Chemother* 52:269-278.

318. Ernst, C. M., P. Staubitz, N. N. Mishra, S. J. Yang, G. Hornig, H. Kalbacher, A. S. Bayer, D. Kraus, and A. Peschel. 2009. The bacterial defensin resistance protein MprF consists of separable domains for lipid lysinylation and antimicrobial peptide repulsion. *PLoS Pathog* 5:e1000660.
319. Cui, L., E. Tominaga, H. M. Neoh, and K. Hiramatsu. 2006. Correlation between Reduced Daptomycin Susceptibility and Vancomycin Resistance in Vancomycin-Intermediate *Staphylococcus aureus*. *Antimicrob Agents Chemother* 50:1079-1082.
320. Julian, K., K. Kosowska-Shick, C. Whitener, M. Roos, H. Labischinski, A. Rubio, L. Parent, L. Ednie, L. Koeth, T. Bogdanovich, and P. C. Appelbaum. 2007. Characterization of a daptomycin-nonsusceptible vancomycin-intermediate *Staphylococcus aureus* strain in a patient with endocarditis. *Antimicrob Agents Chemother* 51:3445-3448.
321. Kaatz, G. W., T. S. Lundstrom, and S. M. Seo. 2006. Mechanisms of daptomycin resistance in *Staphylococcus aureus*. *Int J Antimicrob Agents* 28:280-287.
322. Ernst, C. M., and A. Peschel. 2011. Broad-spectrum antimicrobial peptide resistance by MprF-mediated aminoacylation and flipping of phospholipids. *Mol Microbiol* 80:290-299.
323. Talbot, G. H., J. Bradley, J. E. Edwards, Jr., D. Gilbert, M. Scheld, and J. G. Bartlett. 2006. Bad bugs need drugs: an update on the development pipeline from the Antimicrobial Availability Task Force of the Infectious Diseases Society of America. *Clin Infect Dis* 42:657-668.

324. Giske, C. G., D. L. Monnet, O. Cars, and Y. Carmeli. 2008. Clinical and economic impact of common multidrug-resistant gram-negative bacilli. *Antimicrob Agents Chemother* 52:813-821.
325. Andremont, A., M. Bonten, J. Kluytmans, Y. Carmeli, O. Cars, and S. Harbarth. 2011. Fighting bacterial resistance at the root: need for adapted EMEA guidelines. *Lancet Infect Dis* 11:6-8.
326. Dewan, P. C., A. Anantharaman, V. S. Chauhan, and D. Sahal. 2009. Antimicrobial action of prototypic amphipathic cationic decapeptides and their branched dimers. *Biochemistry* 48:5642-5657.
327. Chopra, I., L. Hesse, and A. J. O'Neill. 2002. Exploiting current understanding of antibiotic action for discovery of new drugs. *J Appl Microbiol* 92 Suppl:4S-15S.
328. Simoni, R. D., and P. W. Postma. 1975. The energetics of bacterial active transport. *Annu Rev Biochem* 44:523-554.
329. Peters, B. M., M. E. Shirtliff, and M. A. Jabra-Rizk. 2010. Antimicrobial peptides: primeval molecules or future drugs? *PLoS Pathog* 6:e1001067.
330. Horton, K. L., and S. O. Kelley. 2009. Engineered apoptosis-inducing peptides with enhanced mitochondrial localization and potency. *J Med Chem* 52:3293-3299.
331. Muthaiyan, A., J. A. Silverman, R. K. Jayaswal, and B. J. Wilkinson. 2008. Transcriptional profiling reveals that daptomycin induces the *Staphylococcus aureus* cell wall stress stimulon and genes responsive to membrane depolarization. *Antimicrob Agents Chemother* 52:980-990.

332. Kilelee, E., A. Pokorny, M. R. Yeaman, and A. S. Bayer. 2010. Lysyl-phosphatidylglycerol attenuates membrane perturbation rather than surface association of the cationic antimicrobial peptide 6W-RP-1 in a model membrane system: implications for daptomycin resistance. *Antimicrob Agents Chemother* 54:4476-4479.
333. dos Santos Mota, J. M., J. A. den Kamp, H. M. Verheij, and L. L. van Deenen. 1970. Phospholipids of *Streptococcus faecalis*. *J Bacteriol* 104:611-619.
334. Cao, M., and J. D. Helmann. 2004. The *Bacillus subtilis* extracytoplasmic-function sigmaX factor regulates modification of the cell envelope and resistance to cationic antimicrobial peptides. *J Bacteriol* 186:1136-1146.
335. Eldholm, V., B. Gutt, O. Johnsborg, R. Bruckner, P. Maurer, R. Hakenbeck, T. Mascher, and L. S. Havarstein. 2010. The pneumococcal cell envelope stress-sensing system LiaFSR is activated by murein hydrolases and lipid II-interacting antibiotics. *J Bacteriol* 192:1761-1773.
336. Wittmann, M., U. Linne, V. Pohlmann, and M. A. Marahiel. 2008. Role of DptE and DptF in the lipidation reaction of daptomycin. *FEBS J* 275:5343-5354.
337. Yang, S. J., Y. Q. Xiong, P. M. Dunman, J. Schrenzel, P. Francois, A. Peschel, and A. S. Bayer. 2009. Regulation of mprF in daptomycin-nonsusceptible *Staphylococcus aureus* strains. *Antimicrob Agents Chemother* 53:2636-2637.

CHAPTER 7: VITA

Danielle McGrath was born in Santa Rosa, California on January 31, 1981. She graduated from Piner High School in June 1999 and began serving her country in the US Navy in January 2000. She completed training as a US Naval Hospital Corpsmen and served until July 2002. Following completion of her active service in the US Navy she received her Associates degree in Mathematics from Glendale Community College in Glendale, Arizona in 2004 followed by her Bachelor's degree in Cellular and Molecular Biology from Northern Arizona University in Flagstaff, Arizona in 2006. She entered the Ph.D. program at the University of Texas Health Science Center in the fall of 2006 and selected to peruse her graduate studies under the mentorship of Dr. Renata Pasqualini, studying the interaction of antimicrobial peptides with the bacterial membrane.

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