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New Approaches to Sensitize Multidrug-Resistant Gram-Negative Bacterial Pathogens to Host Innate Immune Clearance

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

by

Jason Munguia

Committee in charge:

Professor Victor Nizet, Chair
Professor Richard Gallo
Professor John Guatelli
Professor Joseph Pogliano
Professor Sharon Reed

2016

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Chair

University of California, San Diego

2016

DEDICATION

Para toda mi familia, porque sin ustedes, no podria saber donde estaria. Gracias por todo su apoyo.

To my friends, who have not only supported me throughout the process, but also allowed me to keep my sanity during difficult times. Without them I would have become lost in my work and come out a shadow of myself. Instead, I have become a more adventurous and outgoing person even during stressful times.

EPIGRAPH

Intelligence is a valuable thing, but it is not usually the key to survival. Sheer fecundity ... usually counts. The intelligent gorilla doesn't do as well as the less intelligent but more-fecund rat, which doesn't do as well as the still-less-intelligent but still-more-fecund cockroach, which doesn't do as well as the minimally-intelligent but maximally-fecund bacterium.

Isaac Asimov

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Chapter 1 is a portion from an invited review article that will be submitted to *Trends in Pharmacological Sciences* as: Munguia J & Nizet V. Pharmacological Targeting of the Host-Pathogen Interaction: An Alternative to Classical Antibiotics to Combat Drug-Resistant Superbugs. This dissertation author is the primary author of this paper.

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Resistant *Acinetobacter baumannii*. The dissertation author is the primary investigator and author of this paper.

Chapter 3 is a prepared manuscript ready for submission: Munguia J, LaRock D, Tsunemoto H, Olson J, Pogliano J, Nizet V. The Mla Pathway is Critical for *Pseudomonas aeruginosa* Resistance to Outer Membrane Permeabilization and Host Innate Immune Clearance. The dissertation author is the primary investigator and author of this paper

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PUBLICATIONS

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Awakawa, T., Crüsemann, M., **Munguia, J.**, Ziemert, N., Nizet, V., Fenical, W., Moore, B. S. Salinipyrone and Pacificanone Are Biosynthetic By-products of the Rosamicin Polyketide Synthase. *Chembiochem*. 2015 Jul 6; 16(10): 1443–7.

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ABSTRACT OF THE DISSERTATION

New Approaches to Sensitize Multidrug-Resistant Gram-Negative Bacterial Pathogens to Host Innate Immune Clearance

by

Jason Munguia

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2016

Professor Victor Nizet, Chair

Multidrug-resistant Gram-negative (MDRGN) bacterial pathogens pose a significant threat to public health in the US and worldwide. The lack of therapeutic options for MDRGN bacterial infection have forced clinicians to use older, once rarely used antibiotics that are associated with significant toxicity. An increasing number of immunocompromised individuals, including the elderly, premature neonates, surgical patients, organ transplant recipients, and those receiving cancer chemotherapy, represent an expanding reservoir for MDRGN bacteria in the hospital setting. Research to understand the molecular host-pathogen interactions involved in MDRGN bacterial infections and reveal novel therapeutic targets are imperative if we are to prevent the advent of a post-antibiotic era.

For this PhD dissertation project, I began by searching for genes in the MDRGN bacterial pathogen *Acinetobacter baumannii* that are important in resisting the bactericidal action of the human cathelicidin antimicrobial peptide, LL-37, a critical front-line effector of host innate defense. A transposon insertion in *vacJ*, a component of the *mla* system conserved in all Gram-negative species, proved important in mediating resistance to LL-37, detergents, whole blood, complement, and neutrophils. *A. baumannii* lacking *vacJ* demonstrated reduced virulence compared to wild-type parent strain in a murine pneumonia model. To expand the general significance of this work, I examined the virulence capacity of multiple *mla* mutants in *Pseudomonas aeruginosa*, a leading MDRGN opportunistic human pathogen. *P. aeruginosa* lacking an *mla* system exhibited increased sensitivity to LL-37 and its murine homolog, as well as membrane active pharmaceuticals, serum, and whole blood. A murine pneumonia model revealed reduced bacterial burden and mortality when infected with bacteria lacking *vacJ*. Finally, I explored how a common antibiotic, azithromycin, traditionally considered ineffective against MDRGN bacteria in standard testing assays, gained entry to its ribosomal target when endogenous antimicrobial peptides modify the outer membrane, allowing it to provide clear efficacy in an *in vivo* model of *A. baumannii* pneumonia. Altogether, this dissertation furthered our understanding on the importance outer membrane integrity for Gram-negative bacteria to resist host immune clearance. This research points to a possible therapeutic target in the *mla* pathway to control MDRGN infections by sensitizing them to the host innate immune system.

CHAPTER 1

INTRODUCTION

An alarming and persistent rise in antibiotic resistance among many important pathogenic bacterial species poses one of the greatest challenges to the public health. The U.S. Centers for Disease Control and Prevention has estimated that drug-resistant bacteria sicken more than 2 million people annually causing 23,000 deaths, resulting in $20 billion in excess health-care costs and an additional $35 billion in lost productivity. A 2016 U.K. Government "*Review on Antimicrobial Resistance*," in collaboration with the Wellcome Trust, concluded that without a dramatic change in our response to the crisis, the true cost of antimicrobial resistance will be 300 million premature deaths and up to $100 trillion lost to the global economy by 2050, ultimately exceeding cancer as a cause of human mortality. As WHO Director Margaret Chan stated last year: "*With few replacement products in the pipeline, the world is heading towards a post-antibiotic era in which common infections will once again kill. This will be the end of modern medicine as we know it*."

The effects of the antibiotic resistance epidemic are particularly distressing in hospitals and chronic care facilities, where such infections often strike the most vulnerable patient groups with chronic diseases and weakened immune systems. High-risk populations include the elderly, cancer patients, diabetics, premature newborns, individuals who have undergone recent surgery, and those fighting for their lives in the intensive care unit. Ultimately, the impact of antibiotic resistance falls disproportionately on developing countries with poor public health infrastructure that will not be able to deploy expensive last-line antibiotic treatments.

The origins of the current predicament are multifactorial. Overzealous use of traditional broad-spectrum antibiotics in both clinical and agricultural settings, the departure of many major pharmaceutical companies from antibiotic development viewed as unprofitable, and simple Darwinian evolution of microbes exposed to life-or-death selective pressures each contribute

profoundly. Can we, through innovation, lift ourselves out of the hole that we have dug? Changing the very definition of “antibiotic” will be a prerequisite for success.

The scientific basis for current antibiotics centers solely on the bacterium, yet serious infection is more correctly understood as a disease of the host-pathogen interaction. Most leading agents of human bacterial infection typically colonize the skin or mucosal surfaces of healthy individuals without producing symptoms. By definition, if a bacterium has invaded into the bloodstream or deep tissues to produce acute infection, the innate immune system has failed in its sentinel defense function. A more holistic definition of antibiotic therapy that centers on correcting the dysfunctional host-pathogen interaction can unlock opportunities for therapeutic innovation – including drugs with minimal risk of damage to the normal microbiome.

Conventional antibiotics have cured more disease than all other drug classes combined, but this era is coming to a close. Unfortunately, this remarkable success has bred complacency, and increasingly complex patients and multi-resistant pathogens are exacting high morbidity and mortality in the face of a monolithic approach to therapy. By contrast, complacency has never taken hold in the field of cancer therapeutics, where poor prognoses remain for many of the most severe malignancies. Here recent innovative approaches such as inhibition of the overactive tyrosine kinase BCR-Abl in chronic myelogenous leukemia or PDL-1 checkpoint inhibitors to boost antitumor T cells in melanoma have dramatically improved outcomes. They can provide inspiration for future precision medicines that allow us to resist or cure antibiotic resistant infections without destroying our microbiome.

Neutralization of Virulence Factors: Disarming the Pathogen

Virulence factors are those characteristics that differentiate disease-causing bacteria from the hundreds of species of beneficial bacteria that comprise the normal flora of our intestine, mucosal surfaces and skin. Virulence factors include bacterial surface structures or secreted molecules that promote mucosal/epithelial adherence, biofilm formation, and/or intracellular

invasion to breech host cell barriers. Additional virulence determinants promote resistance to immunological clearance by host antimicrobial peptides, complement or phagocytes, thereby allowing the pathogen to continue replication in normally sterile sites and produce a deep-seated or systemic infection. Finally, bacterial factors that directly injure host cells or membranes, impair critical host cell functions, or elicit exaggerated and deleterious pro-inflammatory responses, collectively referred to as “toxins”, are important contributors to virulence.

The basic pharmacological concept at play here is rather than identifying drugs than directly kill or suppress the growth (classical bactericidal or bacteriostatic antibiotic activities), one could screen or design drugs to target a virulence factor of the pathogen, rendering the pathogen “harmless” or readily susceptible to immune clearance. The ideal virulence factor target should be universally or widely expressed among disease-associated strains of the pathogenic bacterial species, and play an essential role in disease pathophysiology. An advantage to the virulence factor neutralization concept is that the therapy is envisioned to be very specific for the pathogen in question, avoiding the extensive “collateral damage” to the normal flora that accompanies courses of broad-spectrum antibiotic therapy and increases the risk of opportunistic infection or metabolic derangements. Virulence factor inhibitor therapy can also be contemplated as a companion or adjunct to effective classical antibiotic therapy (when available) in order to improve clinical outcome in severe or recalcitrant infections, or as a prophylactic therapy for patients entering a high-risk window for nosocomial infections (e.g. chemotherapy, major surgery).

Block epithelial adherence or biofilm formation

An essential first step in disease pathogenesis for many mucosal pathogens is to gain a secure foothold on the epithelium of the target organ, either by specific adhesin-mediated interactions with a host epithelial cell receptor, indirect binding to mucus or extracellular matrix components, or formation of a polymeric self-produced matrix of extracellular substances known as a biofilm. The opportunistic and frequently multidrug-resistant Gram-negative bacterial

pathogen *Pseudomonas aeruginosa* binds to glycostructures on mucosal surfaces via two carbohydrate-binding proteins (lectins), PA-IL and PA-IIL. D-galactose, as well as peptides mimicking human natural killer-1 antigen (HNK-1), polysialic acid or fucose blocked PA-IL-mediated airway cell binding, while L-fucose and pHNK-1 blocked PA-IIL-mediated binding, restoring normal ciliary beat frequency (1). *P. aeruginosa* also produces a fucose-specific lectin, LecB, which participates in tissue attachment and the formation of biofilms. High-affinity LecB ligands obtained by screening combinatorial libraries of multivalent fucosyl-peptide dendrimers induced total dispersion of established biofilms in several clinical *P. aeruginosa* isolates (2). Uropathogenic *Escherichia coli* (UPEC) is the most common cause of urinary tract infections, and its type I fimbrial (FimH) lectin is a key factor in bladder cell adherence and colonization. A small molecular weight orally bioavailable “mannoside” inhibitor that targets FimH in its mannose-binding pocket showed good therapeutic efficacy against UPEC infections, including antibiotic-resistant strains (3). In Gram-positive pathogens such as *Staphylococcus aureus*, many surface proteins, including fibronectin-binding protein A critical for epithelial adherence, are anchored to the cell envelope by the action of transpeptidase enzymes called sortases, which recognize a motif (e.g. LPXTG) in the target protein and catalyze a covalent linkage to cell wall peptidoglycan. Small molecules that exhibit potent inhibitory activity against *S. aureus* sortase A and B *in vitro* showed the capacity to block adherence of the living pathogen to fibronectin (4).

Monoclonal antibodies

The antibiotic resistance crisis has spurred renewed interest in monoclonal antibody (mAb) therapeutics against specific antigens expressed by antibiotic resistant pathogens. Many of these passive immunotherapies function to bind conserved surface targets of the pathogen, which themselves may or may not have a role in virulence, in order to promote opsonization and phagocytic clearance of the organism, as recently reviewed (5, 6). Here we would like to highlight some novel mAb therapeutic concepts that target toxins or other virulence factors of important

pathogens with the goal of improving disease outcome without directly promoting opsonophagocytic clearance/killing of the pathogen.

Alpha-hemolysin ("alpha toxin") of *S. aureus* is a small beta-barrel pore-forming toxin with a broad range of cellular specificities that binds to its receptor (ADAM10) on target cells and triggers membrane disruption (7). Alpha-toxin is an important virulence factor, as demonstrated extensively in by targeted mutagenesis of the encoding *hla* gene in strains of the globally disseminated epidemic USA 300 clone of methicillin-resistant *S. aureus* (MRSA), which markedly reduces host cell injury and virulence potential in murine models of infection, particularly pneumonia (8). Monoclonal antibodies targeting alpha-toxin prevent assembly of its stable oligomer on the target cell surface and protect mice against lethal *S. aureus* pneumonia (9). Treatment with such a mAb was subsequently shown to provide a dose-dependent increase in murine survival across a variety of different *S. aureus* clinical isolates, reduced proinflammatory cytokine and chemokine release, improved lung function independent of Fc activities, and synergistic or additive effects with concurrent antibiotic therapy (10). Two anti-alpha toxin mAbs (MEDI4982/Medimmune and AR301/Aridis) have now entered Phase I/II human clinical trials for prevention of staphylococcal pneumonia (6). *S. aureus* also produces a family of related bi-component leukocidins shown in several studies to make contributions to disease severity: Panton-Valentine leukocidin (PVL), gamma hemolysin (HlgABC) and leukocidin ED (LukED). A recent study described a single human monoclonal antibody capable of recognizing a conformational epitope shared by all of these bi-component staphylococcal toxins plus alpha-toxin, preventing induced lysis of human phagocytes, and providing a high level of protection in murine pneumonia and sepsis models (11).

Another key aspect of *S. aureus* pathogenesis is subversion or dysregulation of the host coagulation system through specific virulence determinants that bind or activate key clotting factors and platelets to promote pathogen clumping, endovascular clot formation and/or development of tissue abscesses. The *S. aureus* surface protein clumping factor A (ClfA) is

documented virulence factor for which a therapeutic mAb has been evaluated in a phase II trial of hospitalized patients with documented *S. aureus* bacteremia. In a recent study, mice systemically challenged with *L. lactis* expressing ClfA succumbed to death within 24 h. Passive immunization of such mice with an anti-ClfA mAb antibody that blocks fibrinogen binding dramatically increased survival compared to a control mAb antibody that bound ClfA but allowed fibrinogen binding (12). This result suggests that inhibition of virulence functions (fibrinogen-binding) and not simply antibody-mediated opsonization contributed to the efficacy of the passive immunization. Coagulase A (CoA) and von Willebrand factor binding protein (vWbp) are two additional *S. aureus* proteins that synergize with ClfA to allow the pathogen to create fibrin cables *in vivo*. In a murine endocarditis model, passive immunization against all three virulence factors (CoA, vWbp, ClfA) reduced the development of heart lesions and increased survival (13). The combination of prothrombin inhibitors along with anti-ClfA mAb also prolonged time to death in *S. aureus* sepsis (13).

Newer mAbs that target bacterial toxins with mechanisms of action other than membrane pore formation have also been shown to improve specific bacterial disease outcomes. The virulence potential of *P. aeruginosa* is largely associated with a type III secretion system (TTSS), a type of molecular syringe that allows the bacteria to directly inject cytotoxins into host cells, inducing cell death. Protein PcrV makes an indispensable contribution to *P. aeruginosa* TTSS toxin translocation, and a molecularly engineered humanized anti-PcrV IgG antigen-binding fragment, KB001, has been developed for clinical use in ventilator associated pneumonia and the chronic pneumonitis of cystic fibrosis (14), with other mAbs targeting PcrV likewise showing efficacy in pre- and post-challenge animal models of *P. aeruginosa* infection (15). Obiltoxaximab, a mAb targeting protective antigen (PA), a component of anthrax lethal toxin (LT) and edema toxin (ET) required for toxin entry, prevents dissemination of *Bacillus anthracis* and reduces mortality in pre- and post-exposure rabbit or cynomolgus macaque models of inhalational anthrax, a foremost biodefense concern (16). A cocktail of one fully human MAb specific to the

receptor binding domain of *Clostridium difficile* toxin A and two fully human MAbs specific to nonoverlapping regions of the glucosyltransferase domain of *C. difficile* toxin B protected hamsters against mortality and reduced severity and duration of diarrhea upon challenge with highly virulent *C. difficile* strains (17). Finally, an interesting derivation of mAb therapeutics was reported in the treatment of difficult *S. aureus* infections, where a slow-growing intracellular reservoir of the pathogen is often resistant to antibiotic clearance. An anti-*S. aureus* mAb was conjugated to a very efficacious antibiotic (rifalogue) that was activated only after it is released in the proteolytic environment of the phagolysosome (18). This unique mAb-drug conjugate showed superior efficacy vs. vancomycin in a mouse model of MRSA bacteremia.

Strategies for toxin neutralization

Beyond specific monoclonal antibodies, other approaches have been devised to prevent the action of bacterial toxins by their physical sequestration or interfering with their key initial interactions with host cell receptors or cellular trafficking pathways.

The essential mechanism of action of bacterial pore-forming toxins reflects their natural affinity for the lipid bilayer of host cell membranes, wherein they assemble to disrupt the integrity of the membrane and promote cell death through hypo-osmotic cell lysis. By providing pharmacological agents that mimic the structural features or biochemical properties of the host cell membrane, a “decoy” strategy can be applied to sequester toxin away from the target cells and protect the host from pathogenic insult. An early example of this utilized cholesterol, packaged within methyl-beta-cyclodextrin (CD), as a topical therapy to sequester alpha-toxin and prevent corneal erosions in a rabbit model of ocular keratitis with live *S. aureus* or purified toxin (19). Another modified CD compound, IB201, effectively blocked alpha-toxin-induced lysis of human alveolar epithelial cells, preventing formation of the lytic pore, and was able to prevent or treat pulmonary infection in the mouse with highly virulent *S. aureus* strains (20). Another group engineered artificial liposomes composed exclusively of naturally occurring lipids tailored to

effectively compete with host cells for toxin binding. Liposome-bound toxins such as staphylococcal alpha-toxin or *Streptococcus pneumoniae* pneumolysin were unable to lyse mammalian cells *in vitro*. Administration of artificial liposomes up to 10 hours after infectious challenged rescued mice from fatal septicemia caused by *S. aureus* and *S. pneumoniae* and protected against invasive pneumococcal pneumonia (21). Another innovative approach derives biomimetic "nanosponges", which consists of a polymeric nanoparticle core surrounded by freshly isolated red blood cell membranes, can function as toxin decoy *in vivo* to scavenge *S. aureus* alpha-toxin and improve survival in toxin-challenged mice (22) or reduce lesion size in a staphylococcal skin infection model (23). Platelet-membrane derived nanosponge mimics have also been shown to bind pathogens and enhance the therapeutic efficacy of vancomycin in systemic *S. aureus* infection when delivered by the platelet-mimetic nanoparticles (24).

Blocking host receptors and entry pathways used by toxins is a promising therapeutic concept, because extensive adaptations by the microbe could be required to enable it to switch to a new receptor that can still support pathogenesis. For example, phage display was used to select a peptide that binds both natural host cell receptors for anthrax toxins (ANTXR1 and ANTXR2), which was subsequently made polyvalent by binding the peptide to a synthetic scaffold, achieving effective neutralization of anthrax toxin action *in vivo* following intravenous administration in rats (25). The cell surface metalloprotease ADAM 10 serves as a receptor for *S. aureus* alpha-toxin, and a small molecule inhibitor of ADAM 10 protease activity protects against alpha-toxin mediated disease pathogenesis in mouse models of staphylococcal pneumonia (8) and skin abscess formation (26). Subsequent to receptor binding, a common mechanism for cellular entry of bacterial toxins requires trafficking to an acidified endosome, which promotes translocation across the host membrane. The most active compound identified in a 30,000 compound screen against anthrax lethal toxin activity, 4-bromobenzaldehyde N-(2,6-dimethylphenyl)semicarbazone (EGA), effectively blocks the entry of anthrax lethal toxin and multiple other acid-dependent bacterial toxins into mammalian cells (27).

Direct inhibition of the enzymatic activity of bacterial protease toxins has also been explored therapeutically. For example, a small molecule hydroxamate compound that inhibits the metalloprotease activity of anthrax LT, provided a significant survival advantage to mice given a lethal challenge of *B. anthracis* vegetative cells or rabbits given a lethal inhalation challenge of *B. anthracis* spores (28). Likewise, ebselen inhibits the cysteine protease activity of *C. difficile* toxins TcdA and TcdB in nanomolar concentrations. Using both the purified toxin and a murine infection model, ebselen administration increased survival of mice as well as reduced pathological injury to the gastrointestinal mucosa (29). Finally, a number of bacterial pathogens that produce pore-forming toxins induce macrophage necroptosis, a highly pro-inflammatory mode of cell death regulated by RIP1 and RIP3 kinases and mediated by the effector MLKL; macrophages deficient in MLKL are consequently resistant to pore-forming toxin induced cell death. Treatment of mice with necrostatin-5, an inhibitor of RIP1 and/or GW806742X, an inhibitor of MLKL, reduced the severity of *Serratia marcescens* pneumonia in a mouse model, protecting alveolar macrophages from cell death and reducing bacterial burden (30); necrostatin-5 also reduced pneumolysin-mediated macrophage necroptosis and cardiac damage following pneumococcal myocardial invasion in a bacteremia model (31).

Reducing bacterial virulence factor gene expression

In most medically important human pathogens, transcription of the genes encoding key virulence determinants or their biosynthetic pathways are regulated by complex, intersecting pathways, which allow fine tuning of their expression in response to multiple environmental cues encountered in the host at each stage of colonization and disease progression. Virstatin is a virulence inhibitor drug contemplated to treat or provide prophylaxis against *Vibrio cholerae* disease that functions to inhibit dimerization of the transcriptional activator, ToxT. When ToxT is blocked, transcription of the genes encoding cholera toxin, which triggers the hallmark watery diarrhea, and the toxin-coregulated pilus (Tcp), which promotes intestinal colonization by the

pathogen, are both shut off (32). Virstatin also inhibits pilus biogenesis, motility and biofilm formation in the multidrug-resistant nosocomial pathogen *Acinetobacter baumannii* (33). Regacin is a drug that inhibits the ability of an AraC-like virulence regulator, RegA, to bind DNA and activate its target promoters in the genome of the model gastrointestinal tract pathogen *Citrobacter rodentium.* Regacin reduced *C. rodentium* toxicity to intestinal epithelial cells, and treatment of mice infected with *C. rodentium* with the drug resulted in reduced colonization and bacterial virulence (34). Similar results have been observed with small molecule inhibitors of VirF, another AraC-like virulence regulator in the human foodborne pathogen *Shigella flexneri*, blocking expression of numerous virulence genes and inhibiting intestinal epithelial cell invasion by the pathogen (35).

S. aureus virulence has also been targeted using the enol-acyl carrier protein inhibitor AFN-1252. Treatment of the pathogen with AFN-1252 rapidly increased the expression of fatty acid synthetic genes, perturbing membrane dynamics in a way that influenced signaling through the SaeRS two-component regulator to repress the expression of virulence genes including alpha-toxin, β- and γ-hemolysins, and two fibrinogen-binding adhesins. Oral treatment of mice with AFN-1252 infected with *S. aureus* in an air pouch model caused a significant reduction in viable bacteria recovered (36). Two small molecule compounds (CCG-2979 and its analog CCG-102487) were identified in a high content screen for reduced expression of streptokinase (SK), a virulence factor of *Streptococcus pyogenes* that co-opts and activates host plasminogen to facilitate systemic spread of the pathogen during invasive infections such as necrotizing fasciitis (“flesh-eating disease”). Microarray analysis of GAS grown in the presence of these inhibitors also showed down-regulation of a number of other important *S. pyogenes* virulence factors in addition to SK, including the antiphagocytic surface anchored M protein and the cytolytic toxins streptolysin O and S, consistent with disruption of a general virulence gene regulatory network. Treatment with the drugs increased neutrophil phagocytosis and killing of *S. pyogenes*, and protected mice from mortality in a systemic infection model (37).

Interference with bacterial quorum sensing

Quorum sensing is an important regulatory principle in numerous bacterial species in which the organisms produce and release chemical signal molecules called autoinducers that increase in concentration as a function of cell density. Many bacterial pathogens use quorum sensing to coordinate gene expression according to the density of their local population within host tissues. The natural product 6-gingerol, a pungent oil derived from fresh ginger, competitively inhibited the binding of two related homoserine lactone autoinducers of *P. aeruginosa* to their cognate receptors, reducing the expression of known virulence factors including elastase and pyocyanin, and blocking biofilm formation. Pre-treatment with 6-gingerol reduced mouse mortality from *P. aeruginosa* infection in a dose-dependent manner (38). Another strategy for competitive inhibition and disruption of *P. aeruginosa* quorum sensing utilized meta-bromo-thiolactone (mBTL), which similarly resulted in reduced biofilm formation, pyocyanin production, and increased survival of *Caenorhabditis elegans* and human lung epithelial cells at low micromolar levels after pseudomonal infection. (39). Using the antimetabolite compound S-phenyl-l-cysteine sulfoxide, another group was able to inhibit the kynurenine pathway implicated in *P. aeruginosa* alkyl quinolone signaling molecule production and quorum sensing, leading to a reduction in the production of pyocyanin and other toxins (40).

In *S. aureus*, the accessory gene regulator (*agr*) quorum sensing system decreases the expression of several cell surface proteins and increases the expression of many secreted toxins in the transition from late-exponential growth to stationary phase. Essential for full *S. aureus* virulence, the *agr* system, and in particular its response regulator AgrA, has begun to be explored as a therapeutic target. Treatment with naringenin, one of the flavonoids present in grapefruits and tomatoes, led to reduced transcription of the *S. aureus* genes encoding AgrA and alpha-toxin, attenuating *S. aureus* alpha-toxin mediated injury to alveolar epithelial cells, and reduced pulmonary inflammation and injury in a mouse pneumonia model (41). Very recently, the

nonsteroidal anti-inflammatory drug diflunisal was also found to block *S. aureus* AgrA transcriptional regulation of small peptide toxin virulence factors known as phenol soluble modulins (PSMs); diflunisal treatment was associated with reduced PSM production and diminished bone destruction in a murine osteomyelitis model of infection (42).

Sensitization of the pathogen to host innate immune clearance

When a patient is suffering a serious infection with a multidrug-resistant pathogen, it is important to consider not only last-line antibiotic options, but also that the pathogen is being combatted by multiple endogenous molecular and cellular effectors of microbial clearance, such as phagocytic cells, reactive oxygen species, and cationic host defense peptides. One emerging concept in alternative infectious disease therapeutics is to identify genes that deprive the pathogen of the virulence factors it uses to defend itself against the host defense mechanisms, thus re-sensitizing it to innate immune destruction. For example, the polysialic acid capsule of K1 serotype *E. coli* provides defense against complement- and phagocyte-mediated innate immune clearance and plays a role in invasive disease potential (e.g. urosepsis). Two small molecule inhibitors identified in a screen for inhibition of *E. coli* capsule biogenesis were recently shown to render the pathogen highly sensitive to active human serum and provided near total protection to mice in a model of lethal systemic infection with a prototypic virulent K1 serotype isolate (43). Recent research has also shown that certain antibiotics that have no demonstrable activity against a particular pathogen in standard laboratory minimum inhibitory concentration (MIC) testing do nevertheless sensitize the pathogen to killing by endogenous host defense peptides, such as human cathelicidin LL-37. Examples include (a) the use of the β-lactam antibiotic nafcillin against MRSA, which promotes LL-37 killing and bacterial clearance in human whole blood, a murine necrotizing skin infection model and human patients with recalcitrant bacteremia (44); and (b) the use of azithromycin to sensitize highly multidrug-resistant Gram-negative bacterial pathogens such as *P. aeruginosa*, *A. baumannii*, *Klebsiella pneumoniae* and

Stenotrophomonas maltophilia to LL-37 killing, human serum and clearance in murine models of pneumonia with each pathogen (45, 46).

Phagocytic cells including neutrophils and macrophages generate reactive oxygen species (ROS) including hydrogen peroxide, hypochlorite and singlet oxygen through the NADPH oxidase-mediated “oxidative burst”, which is a key component of phagolysosomal killing of pathogens after their engulfment. *S. aureus* expresses a hallmark golden carotenoid pigment, staphyloxanthin, with antioxidant properties, which has been shown to promote resistance to ROS and phagocyte killing, increasing virulence in mouse infection models (47). Interestingly, there was a high degree of similarity between a key enzyme required for production of the staphylococcal pigment (CrtM, dehydrosqualene synthase) and a human enzyme (squalene synthase) in the cholesterol biosynthesis pathway. It was discovered that a phosphonosulfate drug BPH-652, a squalene synthase inhibitor in clinical development for its cholesterol lowering properties, blocked *S. aureus* pigment production, sensitizing the pathogen to ROS, neutrophil killing, and reducing the bacterial burden in kidneys of mice following systemic infection (48). Interestingly, the next enzyme in the *S. aureus* carotenoid pigment synthesis pathway is the diapophytoene desaturase, CrtN, which is inhibited by nanomolar concentrations of the FDA approved antifungal drug, naftifine. Pigment inhibition by naftifine treatment led to decreased bacterial burden and increased mouse survival in a systemic *S. aureus* challenge model (49). Blocking carotenoid synthesis also increases cell membrane fluidity to sensitize *S. aureus* to the antimicrobial action of cationic host defense peptides (50).

Statins: Repurposing a leading human medication at the host-pathogen interface

Statins, a family of drugs with a conserved lactone ring structure, bind to the active site of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme involved in cholesterol biosynthesis. Due to their oral availability, favorable pharmacokinetic properties, and relatively low level of associated side effects, these drugs have become a mainstay of LDL

cholesterol-lowering therapy, now used to treat tens of millions of individuals with hyperlipidemia worldwide. Interestingly, recent meta-analyses have compiled a heterogeneous collection of retrospective and prospective cohort studies and randomized trials to conclude that statin use may provide a beneficial effect in reducing morbidity and mortality associated with different infectious disease conditions, including pneumonia, bacteremia, and sepsis (51, 52). Thus it has been hypothesized that statins may have pharmacological effects, either through cholesterol reduction itself or via an independent "off-target" mechanism, to aid in pathogen resistance. Since these drugs have limited direct *in vitro* antimicrobial activity against the leading pathogens, recent studies have focused on the effects of statins on the nature of the host-pathogen interaction.

Statin treatment has been shown to effect the expression of certain key bacterial virulence phenotypes. At subinhibitory concentrations, statins can reduce biofilm formation in *P. aeruginosa*, *S. aureus* and *Staphylococcus epidermidis* (53-55). Simvastatin may also suppressed the production of the *S. aureus* cytolysins, alpha-toxin, and Panton-Valentine leukocidin (54). Various statins have been reported to block host epithelial or endothelial cell invasion by *P. aeruginosa*, *S. aureus*, and the neonatal pathogen group B *Streptococcus* (56-58); the later study providing evidence that an inhibitory action of mevastatin against Rho-family GTPases involved in endocytosis might explain the inhibition of pathogen host cell entry.

Summary

Decreasing treatment options for antibiotic-resistant pathogens places an imperative both on the medical community and upon academic and industry drug discovery initiatives to discover innovative therapeutic approaches to reduce morbidity and mortality in the increasingly complex patient populations at greatest risk. Thinking beyond classical antibiotics to envision strategies that carefully analyze the host-pathogen interaction for opportunities to "tip the balance" back in favor of host immune clearance and preservation of cell and tissue integrity would be a welcome and overdue paradigm shift with the potential for considerable upside. Virulence factor inhibitors

can offer increased specificity and personalization of therapy to the infection at hand, with reduction of undesired side effects placed by broad-spectrum antibiotics on the integrity of the normal host microflora. The definition of virulence determinants for treatment, however, becomes more challenging in immunocompromised patients, where organisms with little *de novo* virulence potential in normal hosts can sometimes produce severe infectious consequences, but much less may be understood about their pathogenic mechanisms.

The many proofs of concept provided illustrate that both screening based and targeted approaches to discovery of virulence factor inhibitors are feasible. Analysis of the biological action of current FDA-approved drugs used for different indications but with known effects on cellular biochemistry and metabolism, offer the opportunity for repurposing as adjunctive antimicrobial agents should they modulate virulence factor expression or fortify host cell resiliency against bacterial toxicity.

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Chapter 1 is a portion from an invited review article that will be submitted to *Trends in Pharmacological Sciences* as: Munguia J & Nizet V. Pharmacological Targeting of the Host-Pathogen Interaction: An Alternative to Classical Antibiotics to Combat Drug-Resistant Superbugs. This dissertation author is the primary author of this paper.

SPECIFIC AIMS

The main purpose of this dissertation is to identify and characterize potentially novel therapeutic targets in Gram-negative bacterial pathogens that would sensitize bacteria to the host innate immune response. I identified increased susceptibility to the human cathelicidin antimicrobial peptide, LL-37, in mutants lacking an Mla pathway, which is conserved across all Gram-negative bacteria. *I hypothesize that the ability to repair and maintain a strong outer membrane is critical for Gram-negative bacteria to resist the actions of host innate immune defense*. I will examine this in the following aims:

Aim 1: Identify bacterial genes responsible for antimicrobial peptide resistance in multidrug-resistant *Acinetobacter baumannii*.

a. Investigate the role of VacJ/Mla pathway in resisting antimicrobial peptides in *A. baumannii*.
b. Characterize the importance of the VacJ/Mla pathway in the context of host innate immune defense to *A. baumannii*.

Aim 2: Characterize the importance of outer membrane integrity in Gram-negative pathogens.

a. Examine the role of VacJ/Mla pathway in *P. aeruginosa* as a potential therapeutic target.
b. Explore how the *in vivo* permeabilization of the Gram-negative outer membrane by host cationic antimicrobial peptides reveals potent bioactivities of azithromycin overlooked in standard antimicrobial testing.

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CHAPTER 2

Mla Pathway Contributes to Antimicrobial Peptide Resistance and Virulence of Multidrug-Resistant *Acinetobacter baumannii*

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Submitted to *mBio*

Running title: Mla pathway in *Acinetobacter* Innate Immune Resistance

PREFACE TO CHAPTER 2

Chapter 2 in full is a manuscript that has been submitted to *mBio*. The work in Chapter 2 addresses Aim 1: Identify bacterial genes responsible for antimicrobial peptide resistance in multidrug-resistant *Acinetobacter baumannii*. A screen of random transposon *A. baumannii* mutants for increased sensitivity to the cathelicidin antimicrobial peptide LL-37 revealed 8 mutants with increased sensitivity. Chapter 2 address Aim 1a: Investigate the role of VacJ/Mla pathway in resisting antimicrobial peptides in *A. baumannii*. This was done by examining the kinetic killing and binding of LL-37 on *A. baumannii* wild-type and *vacJ* mutant. Chapter 2 also address Aim 1b: Characterize the importance of the VacJ/Mla pathway in the context of host innate immune defense to *A. baumannii*. This was accomplished by looking at the effects on *A. baumannii* survival when placed into human whole blood, serum, and in the presence of neutrophils. Furthermore, *A. baumannii* virulence was assessed using a murine pneumonia model of infection and assessed by lung bacterial load, level of bacteremia in the blood, and mouse survival. This work to our knowledge is the first to experimentally examine the importance of VacJ and the Mla pathway in resisting antimicrobial peptides and its necessity for causing full virulence in *A. baumannii* infections.

ABSTRACT

Acinetobacter baumannii is an emerging opportunistic human pathogen in which multidrug resistance severely compromises treatment options leading to significant morbidity and mortality. The mechanisms by which *A. baumannii* establishes an infection and resists the host immune defenses are not completely understood. Here we screened a transposon mutant library of a multidrug-resistant *A. baumannii* strain for hyper-susceptibility to cathelicidin antimicrobial peptide LL-37, a frontline effector of innate immune defense. A transposon that inserted in the gene encoding the *A. baumannii* homologue of VacJ, a lipoprotein in the Mla pathway involved in maintaining outer membrane lipid asymmetry, was identified in this screen. The *A. baumannii vacJ*::Tn*5* (*vacJ*) mutant had no gross defects in growth or morphology, but demonstrated increased binding to LL-37 and faster killing kinetics when exposed to LL-37 compared to the WT parents strain. The *vacJ* mutant has a similar antibiotic susceptibility profile to the parent strain, but showed increased membrane permeability and autolysis in the presence of the detergent sodium dodecyl sulfate. When exposed to human whole blood, serum, or neutrophils, the Mla defective mutant was killed more rapidly than the parent strain. Finally, in an *in vivo* murine model of *A. baumannii* pneumonia, the *vacJ* mutant was markedly attenuated in virulence and survival. This study implicates VacJ and the Mla pathway as potential therapeutic targets that may sensitize *A. baumannii* to killing by host-defenses.

IMPORTANCE

Multidrug-resistant (MDR) *Acinetobacter baumannii* has emerged as a serious threat to human health due to increasing prevalence and decreasing treatment options. By screening a large library of mutant MDR *A. baumannii* mutants, we discovered that VacJ and the Mla pathway play a critical role in the bacteria's ability to resist the host innate immune system. When the Mla pathway is disrupted, there is an increase in the outer membrane permeability of *A. baumannii*, and it becomes more susceptible to killing by human whole blood, serum, and antimicrobial

peptides. When MDR *A. baumannii* is introduced into mouse lungs, more than 80% of the animals are killed after just two days. In stark contrast, when mice are inoculated with *A. baumannii* lacking a functional Mla pathway, there is less inflammation and 75% of the animals survive. Therefore, the Mla pathway represents an interesting therapeutic target that could sensitize the bacteria to killing by the host immune system.

INTRODUCTION

Acinetobacter baumannii is a Gram-negative coccobacillus that has become an important opportunistic pathogen in hospital-acquired infections (1) and an emerging agent in community-acquired infections (2). The most common clinical manifestations associated with *A. baumannii* are ventilator associated pneumonia (VAP) and bacteremia, but the bacterium can also cause catheter-associated urinary tract infections, skin and soft tissue infections, and meningitis (1, 3). Recently, *A. baumannii* has been isolated at alarmingly high rates from serious wound infections of military personnel in the Afghanistan and Iraq conflicts (4). This opportunistic pathogen is notorious for its intrinsic ability to resist many antibiotics and its capacity to readily acquire additional drug resistance mechanisms, greatly complicating successful treatment (5). In the United States, 63% of hospital-acquired *A. baumannii* infections were classified as multi-drug resistant (MDR), sometimes leaving colistin as the only treatment option (6). *A. baumannii* has been highlighted in a select group of drug resistance pathogens known as the ESKAPE pathogens, along with *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, (*Acinetobacter baumannii*), *Pseudomonas aeruginosa*, and the *Enterobacter* species (7), to emphasize their public health significance. Of particular concern, MDR strains of *A. baumannii* are associated with higher mortality rates than more susceptible strains (8). Alarmingly, colistin-resistant strains, though rare, have begun to appear making it imperative for new therapeutic strategies to be developed (9, 10).

The ability of *A. baumannii* to produce serious and even life-threating infections implies an ability to resist the innate immune clearance mechanisms of the hospitalized patients it infects. In contrast to a burgeoning understanding of the genetic basis of *A. baumannii* resistance to pharmaceutical antibiotics (1), the basis of the pathogen's interaction with host innate immunity is only beginning to be explored. To date, a series of well-performed studies have pinpointed *A. baumannii* outer membrane protein A (OmpA) (11), lipopolysaccharide (LPS) (12), the polysaccharide capsule (13), and phospholipase D (14) as bacterial cell components contributing to serum resistance. Further understanding of the pathogen's ability to counteract host immune clearance may provide new therapeutic targets to limit invasive disease pathogenesis.

Among the critical frontline effectors of host innate immune defense are cationic antimicrobial peptides (AMPs) produced by epithelial cells and by circulating and tissue-resident leukocytes are known to protect the host from invasive bacterial infections (15, 16). Cathelicidins are a family of mammalian AMPs with broad-spectrum antimicrobial activity against Gram-negative and Gram-positive bacteria, as well as certain fungi, viruses and protozoan parasites (17). The human cathelicidin AMP, LL-37, is an alpha-helical, amphipathic and anionic peptide that integrates and destabilizes bacterial cell membranes causing their death (18). LL-37 also has important immunomodulatory roles in the proper regulation of cytokine release, chemotaxis, and antigen presentation (reviewed in (19)). Animals deficient in cathelicidin are hyper-susceptible to bacterial infection in multiple organs including skin (20), lung (21), gut (22, 23), brain (24), kidney (25), and eye (26). Essentially by definition, a bacterial pathogen producing significant clinical disease in the human host is displaying some level of functional resistance to endogenous AMPs including cathelicidin (27).

To explore novel mechanisms of *A. baumannii* innate immune resistance, we screened a random transposon mutagenesis library of the clinical *A. baumannii* isolate AB5075 (28) for mutants that were hyper-susceptible to killing by human cathelicidin LL-37. The transposon insertion site in one such mutant mapped to a gene sharing homology to the putative lipoprotein

known as VacJ, which is a part of a pathway (Mla) involved in maintaining outer membrane lipid asymmetry in Gram-negative microorganisms (29). The VacJ family members have been implicated in virulence phenotypes of other Gram-negatives such as in the intracellular spread of *Shigella flexneri* (30, 31), the resistance of *Escherichia coli* to surfactants (32), the serum resistance of non-typeable *Haemophilus influenzae* (33), and resistance of *P. aeruginosa* to pharmaceutical antibiotics (34). Therefore, we further examined the effect of disrupting the Mla pathway in *A. baumannii* on innate immune evasion and virulence phenotypes of this important emerging human disease pathogen.

RESULT

Identification of *A. baumannii vacJ*⁻ mutant in screen for LL-37 hypersensitivity.

We screened a randomly mutagenized transposon Tn*5* library of clinical MDR *A. baumannii* isolate AB5075 (28) for mutants that were sensitized to killing by human cathelicidin LL-37 (at least 2-fold reduction in minimum inhibitory concentration, MIC). Mapping of the chromosomal Tn*5* insertion site in one such hypersensitive *A. baumannii* mutant revealed disruption of a gene encoding a predicted product with strong homology to the putative lipoprotein VacJ of other Gram-negative bacterial species (**Fig. 2.1**), which plays a role in the maintenance of outer membrane lipid asymmetry (29). The abnormal presence of phospholipids in the outer leaflet of the outer membrane has the potential to reduce its barrier function; the Mla pathway restores this asymmetry by retrograde transport of the phospholipids into the inner membrane restoring the stronger barrier created by lipopolysaccharide (LPS) (29). VacJ and the Mla pathway has been associated with varying virulence phenotypes in other Gram-negative bacterial

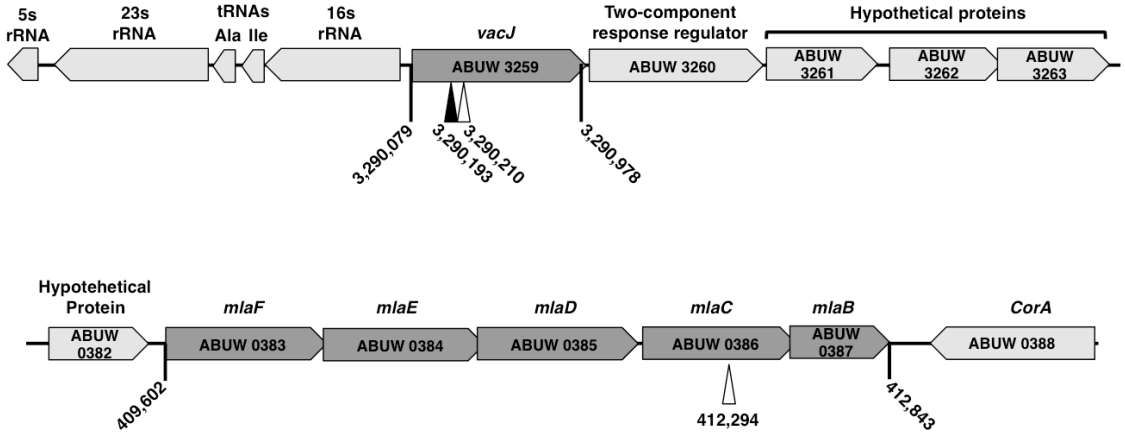


Figure 2.1 Location of transposon in *vacJ* mutant and *mla* genes. Sequencing revealed transposon disrupting an 899 bp gene showing homology to lipoprotein VacJ.

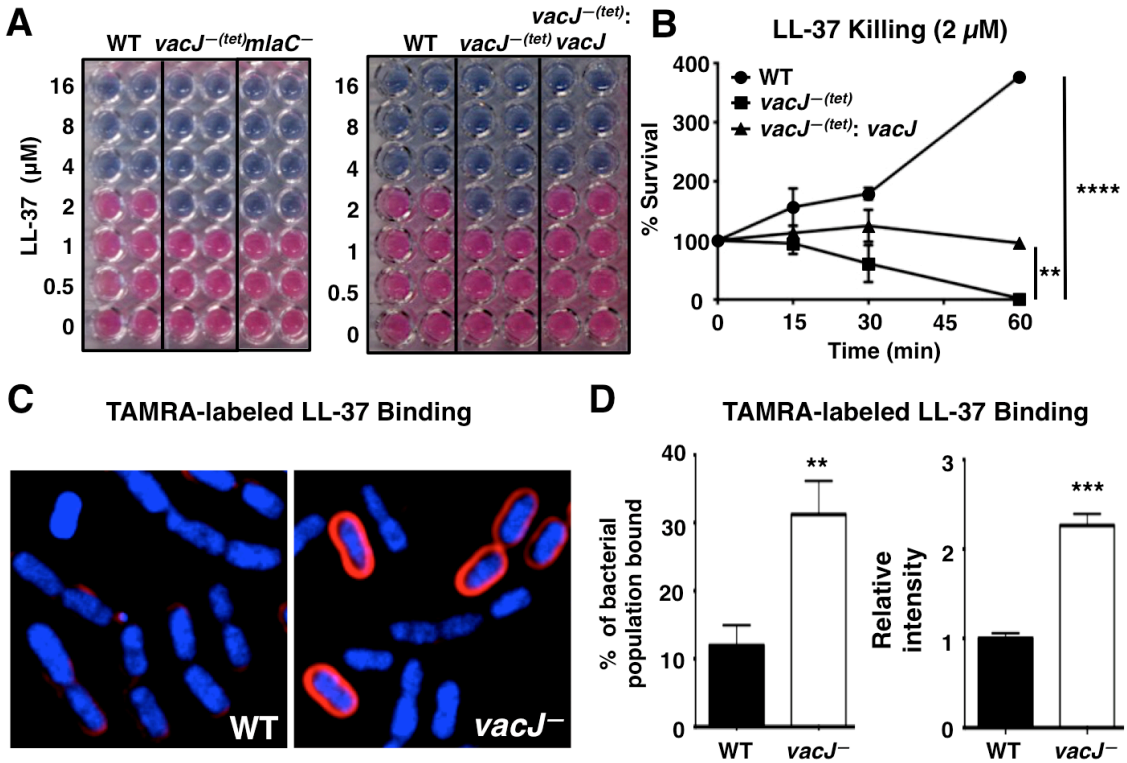


Figure 2.2 Characterizing *A. baumannii* Mla defective mutants sensitivity to LL-37. (**A**) MIC of cathelicidin antimicrobial peptide LL-37 for *A. baumannii* wild-type, *vacJ*, *mlaC*, and complemented *vacJ* mutants. . (**B**) LL-37 killing kinetics at 2 μM LL-37 in RPMI + 5% LB. (**C**) Representative image of TAMRA labeled LL-37 binding to wild-type and *vacJ* mutant (**D**) TAMRA labeled LL-37 binding to *vacJ* mutant and wild-type counterpart were assayed by measuring the average fluorescence intensity per cell and the total percent of the bacterial population bound by LL-37. Data were analyzed by Student's t-test. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Error bars represent standard error of the mean from three independent experiments.

species (30-34); however, the Mla pathway has never been associated with AMP resistance, we investigated loss of function mutants for Mla pathway in *A. baumannii*.

Loss of function Mla mutants in *A. baumannii* are hypersusceptible to and binds more LL-37.

To verify that the *A. baumannii* Mla mutants were more sensitive to cathelicidin killing, formal minimum inhibitory concentrations (MIC) testing was performed on mid-logarithmic phase bacteria using recombinant LL-37 at concentrations ranging from 16 μM to 0.5 μM. The *vacJ*$^-$ and *mlaC*$^-$ mutants exhibited a consistent two-fold decreased MIC (2 μM) compared to the WT parent *A. baumannii* strain (4 μM) (**Fig. 2.2A**); complementation of the *vacJ*$^-$ mutant with a single copy insertion into the genome restored the MIC to WT levels (**Fig. 2.2A**). Since we saw similar LL-37 sensitivity in the *mlaC*$^-$ mutant, we proceeded to investigate the importance of the Mla pathway with the *vacJ*$^-$ mutant. *A. baumannii vacJ*$^-$ displayed accelerated kinetics of bacterial killing at 2 μM LL-37 with the *vacJ* complemented mutant restoring some of the ability to resist killing by LL-37 (**Fig. 2.2B**). Increased LL-37 sensitivity of the *A. baumannii vacJ*$^-$ mutant was correlated to a significant increase in the amount of TAMRA-labeled LL-37 associated with the bacterial cell membrane (**Fig. 2.2C**), as quantified by the intensity of fluorescent staining of each bacterium and the total percentage of the bacterial population stained (**Fig. 2.2D**).

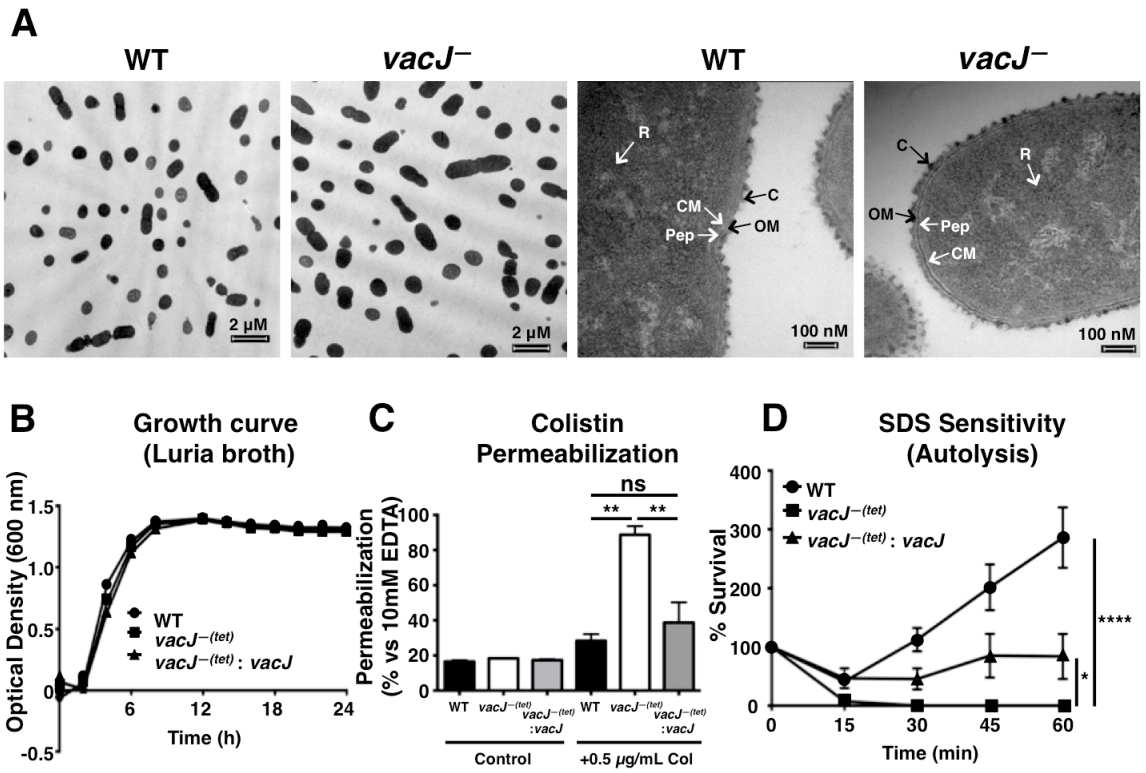


Figure 2.3 *A. baumannii vacJ*⁻ mutant morphology and susceptibility to cell wall stressors. (**A**) TEM analysis of wild type and *vacJ*⁻ mutant bacterial morphology. (**B**) Growth kinetics of *A. baumannii* wild type, *vacJ*⁻ mutant, and complement over 24 h measured at OD_{600}. (**C**) Percent permeability of *A. baumannii* wild-type, *vacJ*⁻ mutant, and complemented bacteria to NPN using 10 mM EDTA treated bacteria as a fully permeabilized standard. Permeability of the wild type, *vacJ*⁻, and complemented mutant was also assessed in the presence of sub-MIC colistin. (**D**) Time kill curves of *A. baumannii* wild type, *vacJ*⁻ mutant, and complement in the presence of 0.03%. R = Ribosomes, CM = Cell membrane, Pep = Peptidoglycan layer, OM = Outer Membrane, C = Capsule. Data were analyzed by Student's t-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Error bars represent standard error of the mean of the results of three independent experiments, with the exception of the NPN assay, which was performed twice.

Analysis of *A. baumannii vacJ*⁻ mutant morphology and susceptibility to cell wall stress.

The *A. baumannii vacJ*⁻ mutant did not exhibit any gross ultrastructural abnormalities as examined by transmission electron microscopy (**Fig. 2.3A**), and no significant changes in the growth rate of the *vacJ*⁻ mutant vs. the wild-type parent strain and the complement were observed in standard Luria broth culture (**Fig. 2.3B**). Thus the *vacJ*::Tn*5* insertion mutant is associated with an increase in sensitivity to LL-37 without causing a fundamental aberration on *A. baumannii* growth or morphology. Lipoprotein VacJ participates in maintaining lipid asymmetry in Gram-negative bacteria by transporting phospholipids present on the outer leaflet of the outer

membrane to the inner membrane (29). An asymmetric lipid distribution in the outer membrane is proposed to provide a stronger cell membrane barrier due to strong lateral interactions of LPS molecules with one another coupled with the low fluidity of the hydrocarbon domain (35). Whenever the LPS barrier is damaged or disrupted, phospholipids translocate into these areas leading to increased permeability of the outer membrane. To determine if the outer membrane of *A. baumannii vacJ* mutant was inherently more permeable, we used the probe 1-N-phenylnapthylamine (NPN), which becomes fluorescent upon binding to the inner membrane of Gram-negative bacteria (36). When exposed to the same concentration of NPN, we saw no significant difference between WT, *vacJ*, and *vacJ* complemented strains (**Fig. 2.3C**). Furthermore, when we subjected the bacterial outer membrane to further stress using sub-MIC concentrations of colistin, a three-fold increase in permeability of the *vacJ* mutant was observed compared to the parent strain (**Fig. 2.3C**). Complementation of the *vacJ* mutant led to the restoration of membrane integrity to near wild-type levels. The *A. baumannii vacJ* mutant also had markedly increased sensitivity to autolysis upon exposure to sodium dodecyl sulfate (SDS) (**Fig. 2.3D**), even in the absence of EDTA chelation, which is required to destabilize outer membrane LPS to promote SDS sensitivity in *E. coli* Mla pathway mutants (29). Complementation of the mutant led to partial recovery of the SDS sensitivity.

Table 2.1 Comparison of wild-type *Acinetobacter baumannii* and isogenic *vacJ* mutant for minimum inhibitory concentrations of a wide panel of antibiotics.

	MIC			MIC			MIC	
Antimicrobial	**WT**	***vacJ***	**Antimicrobial**	**WT**	***vacJ***	**Antimicrobial**	**WT**	***vacJ***
Ampicillin	≥ 32	≥ 32	**Cefpodoxime**	≥ 8	≥ 8	**Gentamicin**	≥ 16	8
Amoxicillin/ Clavulonate	≥ 32	≥ 32	**Cefotaxime**	≥ 64	≥ 64	**Tobramycin**	8	8
Ampicillin/ Sulbactam	≥ 32	≥ 32	**Ceftazidime**	≥ 64	≥ 64	**Nalidixic Acid**	≥ 32	≥ 32
Ticarcillin	≥ 128	≥ 128	**Ceftizoxime**	≥ 64	≥ 64	**Ciprofloxacin**	≥ 4	≥ 4
Piperacillin	≥ 128	≥ 128	**Ceftriaxone**	≥ 64	≥ 64	**Levofloxacin**	4	4
Piperacillin/ Tazobactam	≥ 128	≥ 128	**Cefepime**	≥ 64	≥ 64	**Moxifoxacin**	≥ 8	≥ 8
Cefalotin	≥ 64	≥ 64	**Aztreonam**	≥ 64	≥ 64	**Norfloxacin**	≥ 16	≥ 16
Cefazolin	≥ 64	≥ 64	**Doripenom**	≥ 8	≥ 8	**Tetracycline**	< 1	2
Cefuroxime	≥ 64	≥ 64	**Imipenem**	≥ 16	8	**Tigecycline**	< 0.5	< 0.5
Cefuroxime Axetil	≥ 64	≥ 64	**Meropenem**	≥ 16	8	**Nitrofurantoin**	> 512	> 512
Cefotetan	≥ 64	≥ 64	**Amikacin**	≥ 64	32	**Trimethoprim/ Sulfamethoxazole**	> 320	> 320

A. baumannii vacJ **mutant susceptibility to various antibiotic agents.**

A mutation of the *vacJ* homologue in *P. aeruginosa* was associated with increased susceptibility to antibiotics of different structural classes, including ticarcillin, ciprofloxacin and chloramphenicol (34). In contrast, when comparing the wild-type and *vacJ* mutant *A. baumannii* for antibiotic susceptibility to a large clinical panel of 33 antibiotics spanning beta-lactams (including ticarcillin), fluoroquinolones (including ciprofloxacin), aminoglycosides, tetracyclines, carbapenems, nitrofurantoin and trimethoprim-sulfamethoxizole (**Table 2.1**), no increased susceptibility of the mutant strain was identified. Selected extended antibiotic testing (**Table 2.2**) also showed no change in susceptibility of the *A. buamannii vacJ* mutant to colistin or polymixin B, but corroborated a two-fold increase in susceptibility to chloramphenicol mirroring the finding reported in *P. aeruginosa* (34). We also observed a two-fold increase in sensitivity to very high concentrations (> 10-20 mg/mL) of lysozyme, a muramidase of the innate immune system present in neutrophil granules and mucosal secretions, but the concentrations required for any activity against *A. baumannii* are likely supraphysiologic. In sum, the observed outer membrane permeability defect and increased sensitivity to cathelicidin LL-37 of the *A. baumannii vacJ*

mutant was not associated with global sensitivity to all cell wall active and other antimicrobial agents, but rather exhibits some selectivity.

Table 2.2 MICs of additional antimicrobial agents and peptides.

Antimicrobial	WT *A. baumannii*	*vacJ*- mutant
Cathelicidin LL-37	4 μM	2 μM
mCRAMP	8 μM	4 μM
Polymixin B	1 μg/mL	1 μg/mL
Colistin	2 μg/mL	2 μg/mL
Lysozyme	24 mg/mL	12 mg/mL
Chloramphenicol	32 μg/mL	16 μg/mL
SDS	10 mg/mL	0.3 mg/mL

The *A. baumannii vacJ*- mutant is more susceptible to killing in human whole blood, serum, and by neutrophils.

A. baumannii are associated with bloodstream infections associated with the third highest crude mortality rate in the intensive care unit (37). We extended our investigation to determine the requirement of the Mla pathway in *A. baumannii* resistance to clearance by human blood and blood components. Whereas the wild-type parent *A. baumannii* strain was able to persist in 90% freshly isolated whole human blood, the *vacJ*- mutant was quickly killed with a nearly 2-log reduction in recoverable colony forming units (CFU) within the first 30 min of exposure (**Fig. 2.4A**). The *vacJ*- mutant was also sensitive to killing in 80% human serum, but could survive in heat-inactivated serum, suggesting increased susceptibility to heat-labile complement components (**Fig. 2.4B**). We also exposed *A. baumannii* to freshly isolated human neutrophils at a multiplicity of infection (MOI) = 1 bacteria/neutrophil, and once again observed that the wild-type strain could survive and proliferate whereas the growth of the *vacJ*- mutant was suppressed (**Fig. 2.4C**). These studies demonstrate that the changes in the *A. baumannii* cell membrane associated with loss of function of the Mla pathway sensitize the bacterium to immune components in human blood important for pathogen clearance.

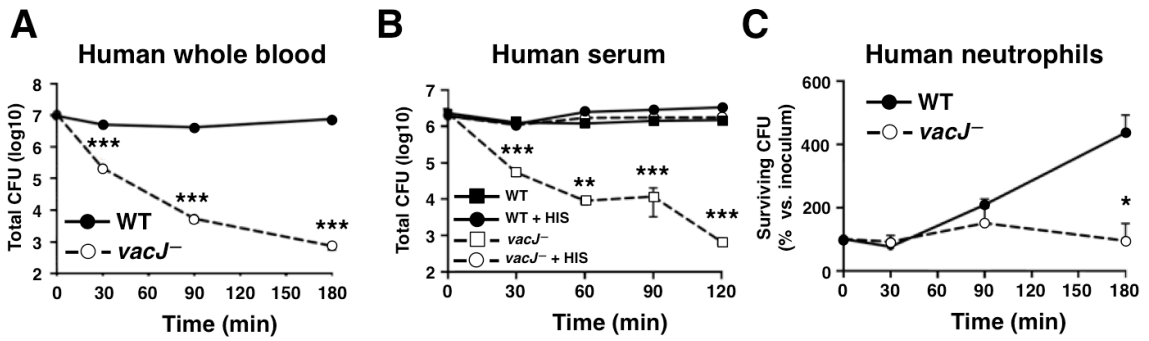


Figure 2.4 *A. baumannii vacJ* susceptibility to human whole blood and blood components. (**A**) Timed kill curve of 10^7 CFU of *A. baumannii* wild-type or *vacJ* added to 90% whole blood. (**B**) Timed kill curve of 10^7 CFU of *A. baumannii* wild-type or *vacJ* added to 80% human serum (squares) and 80% heat inactivated human serum (HIS) (circles). (**C**) Time kill curve of *A. baumannii* wild-type versus *vacJ* mutant upon exposure to PMNs at a multiplicity of infection (MOI) of 1.0 bacteria/neutrophil. Data were analyzed by Student's t-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Error bars represent standard error of the mean of the results of performed in triplicates. Figures are representative of three individual experiments.

***A. baumannii* Mla pathway is essential for full animal virulence in a murine pneumonia model.**

Based on the *ex vivo* studies of human blood and blood components, we predicted VacJ and the Mla pathway could contribute to *A. baumannii* virulence in an animal infection model. A previous study using a library of transposon mutants to uncover genes required for lung infection persistence revealed *vacJ* as an important gene in *A. baumannii* infections(38). As *A. baumannii* is associated with VAP in the hospital setting, we elected to compare the wild type and *vacJ* mutant strains in a murine pneumonia model of infection. Mice were infected intratracheally with 1 x 10^8 CFU of logarithmic phase *A. baumannii* resuspended in PBS in a volume of 30 µl. In a first series of analyses, animals were euthanized 24 hours post infection, and it was observed that animals infected with the WT parent *A. baumannii* strain showed significant pathologic findings in microscopic examination of hematoxylin/eosin sections, including neutrophilic infiltrate and proteinaceous exudate consolidating the alveolar spaces; changes that markedly diminished in animals infected with the *vacJ* mutant (**Fig. 2.5A**). When we enumerated surviving bacteria at the 24 h time point, animals infected with the *A. baumannii* wild-type strain showed a 2-fold

increase in recovered CFU compared to initial inoculum, while animals infected with the *vacJ*⁻ mutant had a 10-fold reduction in bacterial load (**Fig. 2.5B**). Blood was collected simultaneously from the cheeks of animals revealing that majority of the wild type *A. baumannii*-infected mice had high levels of recoverable bacteria in the blood, whereas no *A. baumannii* was recovered from any of the mice infected with the *vacJ*⁻ mutant (**Fig. 2.5C**). Finally, in a separate experiment to assess survival in the murine lung infection model, the kinetics of killing and overall mortality in the wild-type *A. baumannii*-infected group (67% mortality at 36 h, 83% mortality at 5 d) were significantly increased compared to the *vacJ*⁻ mutant-infected group (16% at 36 h, 25% at 5 d) (**Fig. 2.5D**). Thus, the Mla pathway is essential for full animal virulence of *A. baumannii* in a mouse pneumonia model.

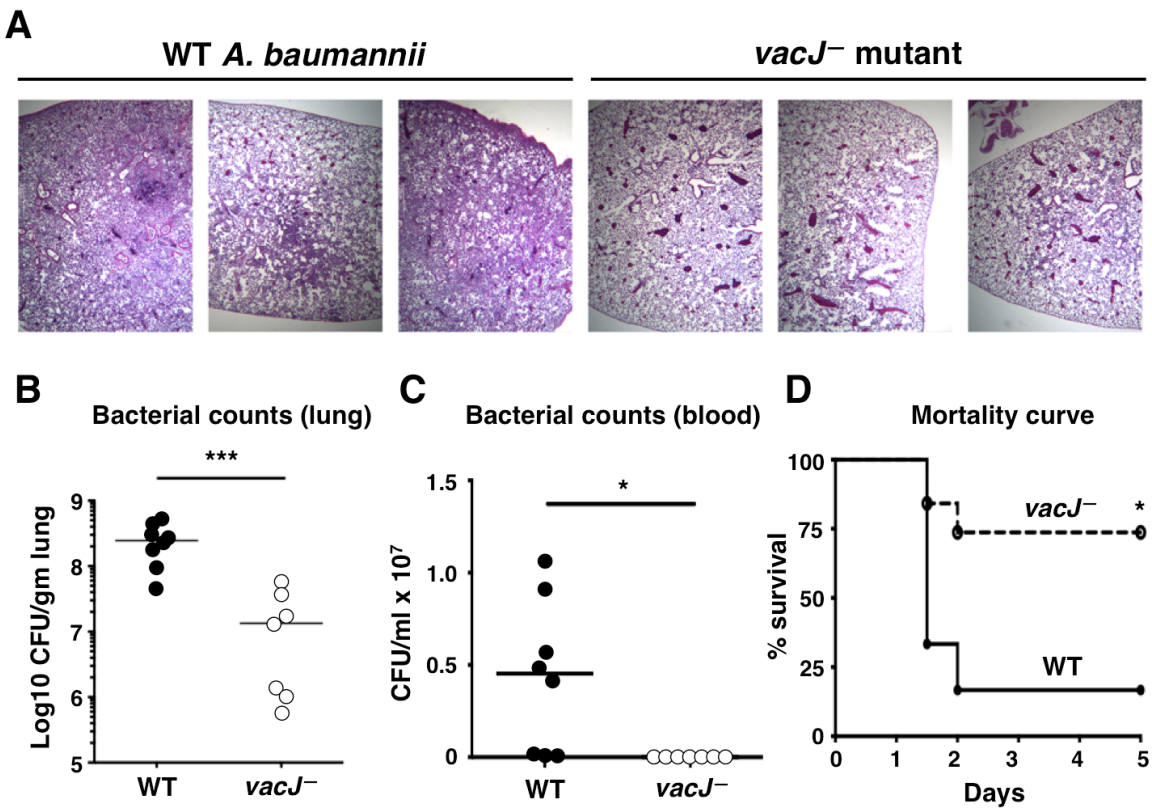


Figure 2.5 *A. baumannii vacJ*⁻ has reduced virulence in murine pneumonia model of infection. (**A**) C57BL/6 mice were intratracheally infected with 10^8 CFU of either *A. baumannii* wild-type or *vacJ*⁻ mutant. Lungs were harvested 24 hours post infection (HPI). The right lobe was used for histopathological analysis and the left lobe was serially diluted and plated for CFU enumeration. H&E staining of formaldehyde fixed sections demonstrating the amount of inflammatory cell infiltrate and disruption to normal lung tissue architecture. (**B**) Number of viable CFU per gram of harvested lung tissue from mice infected with *A. baumannii* wild-type versus the *vacJ*⁻ mutant. *n* = 8 mice per group, except 7 mice in *vacJ*⁻ infection. (**C**) Concentration of viable *A. baumannii* wild-type versus *vacJ*⁻ mutant in the blood of intra-tracheally infected mice 24 HPI; *n* = 8 mice per group, except 7 mice in *vacJ*⁻ infection. (**D**) Mice were infected with $5x10^7$ CFU of either *A. baumannii* wild-type or *vacJ*⁻ mutant and monitored every 12 h for 5 d; *n* = 19 mice per group, except 18 mice in WT infection. * $P < 0.05$, *** $P < 0.001$ determined by two-tailed Mann-Whitney test in lung and blood CFU enumeration. * $P < 0.05$ determined by Log-ranked (Mantel-Cox) test in survival studies.

DISCUSSION

Our medical community and public health infrastructure is experiencing a worrisome and steady rise of difficult to treat multidrug-resistant infections, concomitant with a decrease in novel antibiotics entering clinical trials (7). Of special concern are those infections caused by MDR Gram-negative bacteria due to the limited, and often toxic antibiotics of last resort, and the inherent ability of these opportunistic pathogens to as acquire new resistance phenotypes through horizontal gene transfer (7, 39, 40).

A. baumannii causes nosocomial infections associated with high rates of patient morbidity and mortality (3). Rates of community acquired *A. baumannii* pneumonia have also been on the rise, especially in South East Asia (41), and this pathogen has proven to be a major issue in soldiers with combat-related trauma wounds obtained in the recent military conflicts in Iraq and Afghanistan (4). Although less common, community-acquired *A. baumannii* pneumonia is not strongly associated with extensive antimicrobial resistance to date (42), but the sharp rise of *A. baumannii* multidrug-resistance in the hospital threatens to spread into the communities (43). Finding new therapeutic strategies for MDR *A. baumannii* infections is therefore a medical imperative.

Here, we screened a mutant library of a highly virulent MDR *A. baumannii* strain for increased sensitivity to cathelicidin peptides, and identified a hypersusceptible mutant harboring a disruption in the *vacJ* gene of the Mla pathway. The *A. baumannii* mutants lacking functional Mla pathway demonstrated increased sensitivity to innate immune system components including cathelicidin peptides, serum, neutrophils and whole blood. The innate immune sensitization of the *vacJ* mutant was not accompanied by significant alterations in antibiotic susceptibility, growth or gross ultrastructural morphology. Nevertheless, the *A. baumannii vacJ* mutant was more easily cleared, induced less lung inflammatory changes, and had attenuated virulence in a murine model of pneumonia.

These effects of the *vacJ* disruption suggest that the Mla pathway to which it belongs could represent an interesting pharmacological target for novel antibiotic therapeutics. By impairing the ability of Gram-negative bacteria to maintain lipid asymmetry and repair outer membrane damage, the organisms could be sensitized to killing by the host innate immune system. This approach could fit into a new virulence factor-based paradigm for treating bacterial infections, wherein non-invasive commensal pathogens would remain unharmed (Mla pathway is not essential for growth), while invasive pathogens that activated and were exposed to host antimicrobial peptides, serum, or phagocytes could be sensitized to immune clearance. Core components of the Mla pathway are found in all Gram-negative bacteria; therefore, a VacJ or similar Mla pathway targeted inhibitor potentially provide broad against other invasive or opportunistic Gram-negative pathogens (44). Indeed, a recent publication demonstrated that antibodies against recombinant VacJ from *Pasteurella multocida* (45) provided 50-67% protection as a vaccine antigen in mice. This finding suggests that VacJ is accessible to IgG antibodies and validates one method to inhibiting the Mla pathway.

MATERIALS AND METHODS

Bacteria and genetic manipulations.

A. baumannii strain AB5075, along with a transposon mutagenized pool, were previously described (28) A. baumannii *vacJ*::Tn5 Tet (used for complementation) and *mlaC*::Tn5 Tet mutant were acquired from Colin Manoil. *A. baumannii* was propagated in Luria broth (LB) at 37 ˚C with shaking. For infection studies, bacteria were cultivated at 37˚C to mid-exponential phase (OD_{600nm} = 0.4), followed by serial dilution and enumeration of CFU to verify correct experimental inoculums. For screening, single colonies of transposon mutagenized *A. baumannii* were plated, picked and inoculated into LB in 96-well plates. Plates were grown at 37 ˚C while shaking. Glycerol stocks were made by adding glycerol to a final concentration of 40% and stored at -80 ˚C. Complementation was performed as described (28). Briefly, vacJ gene was cloned into

pUC18T-mini-Tn7T-*hph* using the following primers to amplify vacJ and upstream region into plasmid: Tn7_vacJ_F (GCATGAGCTCACTAGTGGATCCGATGTGCATTATAGACCATCTCTCTC), Tn7_vacJ_R (GTTTGGAACTAGATTTCACTTATCTGGTTATTTTTCGGTTTTATCAGTGTTATCTTC), vacJ_Tn7_F (GAGAGAGATGGTCTATAATGCACATCGGATCCACTAGTGAGCTCATGC) vacJ_Tn7_R (GAAGATAACACTGATAAAACCGAAAAATAACCAGATAAGTGAAATCTAGTTCCAAAC). PCR products were combined 1:1 and electroporated into *E. coli* TOP10 cells selected on Luria broth agar plates containing 100 μg/mL Hygromycin B.

Antibiotics and antimicrobial peptides.

For *in vitro* studies, colistin sulfate was purchased from Sigma-Aldrich; stock solutions were prepared in phosphate buffered saline (PBS) at 10,000 mg/L and frozen at -80 ºC. LL-37 and TAMRA-tagged LL-37 were purchased from the American Peptide Company; stock solutions were prepared in molecular quality water at 640 μM and 320 μM, respectively, and stored at -80 °C.

Sub-MIC LL-37 selection.

A. baumannii transposon mutants were selected by diluting overnight cultures grown in LB 1:100 into 200 μL RPMI + 5% LB and grown to mid-exponential phase. Cultures were then diluted 1:100 into 200 μL of RPMI + 5% LB containing 2 μM LL-37, and 96-well plates were placed at 37 ˚C overnight to monitor growth. Bacterial viability was confirmed by adding 20 μL of resazurin (final concentration 6.75 μg/mL) to cultures and observing color change 8 h later. Selected transposon mutants were verified to be sensitive to LL-37 by performing a formal MIC assay using 5 x 10^5 CFU across a range of concentrations in RPMI + 5% LB. Mutants were plated on LB agar plates with hygromycin B (250 μg/mL) to confirm the presence of the *hyg* cassette in the transposon.

Transposon insertion location.

Transposon insertions were determined by a two-stage semi-degenerate PCR with submission of the PCR product for sequencing (GENEWIZ, Inc. San Diego, CA). Results were then BLASTed to the AB5075 genome(46) as well as *A. baumannii* reference strains to determine gene homology. In the first round of PCR, primer hyg-174 (GAAGCATTTATCAGGGTTATTGTCTCA) was paired with primers CEKG 2A (GGCCACGCGTCGACTAGTACNNNNNNNNNNAGAG), 2G (GGCCACGCGTCGACTAGTACNNNNNNNNNNCTAG), and 2H (GGCCACGCGTCGACTAGTACNNNNNNNNNNTAAGT) along with heat killed bacterial culture in water. In the second round of PCR, products from the first round of PCR were used as a template along with primers hyg-154 (TGTCTCATGAGCGGATACATATTTGA) and CEKG4 (CCGGTGCGCAGCTGATCATG). Products of the second round of PCR were then cleaned up using a Qiagen PCR cleanup kit and submitted for sequencing with primer hyg-107 (AATAGGGGTTCCGCGCACATT).

Electron microscopy of bacterial gross morphology.

A. baumannii cultures were grown overnight in LB at 37 °C with shaking, and PBS stocks prepared by washing twice with PBS via centrifugation at 3,220 x *g* at room temperature with a final resuspension in PBS to an OD_{600} of 0.40. Next, 2.5 mL of *A. baumannii* cultures were added to 47.5 mL RPMI + 5% LB media pre-warmed to 37 °C. Fifty ml cultures were placed in a shaking incubator at 37 °C for 2 h then centrifuged at 3,220 x *g* at room temperature for 10 min. Samples were prepared for viewing as previously described (47). Grids were viewed using a Tecnai G2 Spirit BioTWIN transmission electron microscope and photographs were taken equipped with an Eagle 4k HS digital camera (FEI).

NPN bacterial outer membrane permeability assay.

Assay was performed as previously described (47). Briefly, overnight cultures of *A. baumannii* were grown in LB at 37 °C with shaking were washed twice with PBS via centrifugation at 3,220 x *g* at room temperature and resuspended to an OD_{600} = 0.40 in 4 mL of RPMI + 5% LB for colistin assays. Colistin was added to a final concentration of 0.5 μg/mL to a subset of the portion of the bacterial cultures. The cultures were shaken at 37 °C for 1 h, spun at 3,000 x *g* at room temperature for 5 min, and resuspended in 0.5 mL of 10mM Tris buffer pH 8.0. The concentrated 0.5 mL cultures were used to prepare 2 mL bacterial stocks at OD_{600} 0.40 in 10 mM Tris. Assays were conducted in a final volume of 200 μL in triplicate in 96-well round bottom plates (Costar). Plates were immediately read in a fluorescent plate reader: excitation 250 nm & emission 420 nm.

Sodium dodecyl sulfate (SDS) killing kinetics.

A. baumannii overnight cultures were washed twice with PBS and grown to mid-log phase in fresh LB. Bacterial cultures were then diluted 1:100 and sterile 10% SDS in H_2O was added to make final concentration 0.03%. Time points were collected at zero minute and every 15 min for 1 h, serially diluted, and plated on LB agar plates.

Fluorescent microscopy for TAMRA-LL-37 binding.

Overnight *A. baumannii* cultures were grown in RPMI + 5% LB to mid log phase (OD_{600} 0.4), pelleted, washed twice with PBS, and resuspended to equal volume of RPMI + 5% LB containing 4 μM TAMRA-tagged LL-37. Cultures were placed on a rocker at 37 °C, collected after 30 min, and stained with 1 µg/mL FM4-64 + 2 µg/mL DAPI (Molecular Probes). Stained cultures were centrifuged at 3,300 x *g* for 30 s in microcentrifuge and resuspended in approximately 5% of the original volume. Three µl of concentrated cells were transferred onto an agarose pad containing 1.2% agarose + 20% LB medium for microscopy. Exposure time of each excitation

was the same for each experimental replicate included in the statistical analysis of all treatments. All images (nondeconvolved) were analyzed by ImageJ v1.48f and CellProfiler2.0. Average DAPI or TAMRA intensity per cell was measured then subtracted by their respective background intensities.

Whole blood and serum killing.

Heparinized blood was collected from healthy volunteers and placed in siliconized tubes. 10^7 CFU of *A. baumannii* in RPMI was added so that the final concentration of whole blood was 90%. Samples were taken at time 0, 30, 90, and 180 min, serially diluted, and plated on LB agar plates for CFU enumeration. Serum was collected from healthy volunteers by collecting blood in vacuum-sealed containers (BD) and spun at 300 x *g* for 10 min to separate serum from erythrocytes and other cells. Collected serum was pooled and split; half was heat-inactivated at 56 ˚C for one h while the other half was stored at 4 ºC for 1 h. Serum was partitioned into siliconized tubes and 1 x 10^7 CFU of *A. baumannii* wild-type or *vacJ* mutant in RPMI was added to a final serum concentration of 80%. Samples were collected at 0, 30, 60, 90, and 120 min, serially diluted, and plated on LB agar for enumeration. Experiments were performed in triplicate.

Neutrophil killing.

Heparinized blood was placed on top of polymorph prep and spun at 600 x g for 30 min, without braking, to isolate neutrophils. The neutrophil layer was then collected, washed twice with water to eliminate erythrocytes, spun down at 400 x *g* for 10 min (no brakes), and resuspended in PBS for counting. *A. baumannii* wild-type and *vacJ* mutant bacteria were opsonized in RPMI + 2% human serum for 10 min at 37 ˚C. 1 x 10^6 neutrophils were then combined with 1 x 10^6 CFU of *A. baumannii* in siliconized tubes in RPMI + 2% human serum. Tubes were rotated at 37 ˚C and samples were collected at 30, 90 and 180 min. Neutrophils were lysed by adding Triton X-

100 to a final concentration is 0.025%, triturated, serially diluted, and plated on LB agar for enumeration. Experiments were performed in triplicate.

Murine pneumonia model.

8-week-old female C57BL/6J mice (Jackson Labs) were used for following experiments. *A. baumannii* cultures were grown overnight in LB at 37 °C with shaking, washed twice with PBS, diluted 1:100 in fresh LB and grown to mid log phase. Bacteria were washed twice with PBS via centrifugation at 3,220 x *g* at room temperature and concentrated in PBS to yield appropriate inoculum. For histopathology, lung bacterial load, and blood bacterial load, 1 x10^8 CFU per 30 μL was used to infect the mice (WT infection n = 8, *vacJ* infection n = 7). Mice were anesthetized with 100 mg/kg ketamine and 10mg/kg xylazine. Once sedated, the vocal chords were visualized using an operating otoscope (Welch Allyn) and 30 μL of bacteria or PBS were instilled into the trachea during inspiration using a plastic gel loading pipette tip. Mice were placed on a warmed pad for recovery. After 24 h, mice were sacrificed with CO_2 and lungs were collected for enumeration of the bacteria in the lungs and histopathological analysis. To enumerate the total number of bacteria remaining in the lungs, left lung lobes were removed and placed in a 2 mL sterile micro tube (Sarstedt) containing 1 mL of PBS and 1 mm silica beads (Biospec). Lungs were homogenized by shaking at 6,000 rpm for 30 s (x3) using a MagNA Lyser (Roche), with the specimens placed on ice as soon as they were harvested. Aliquots from each tube were serially diluted for enumeration on LB agar plates. For the *A. baumannii* survival experiment, 5 x 10^7 CFU were given in 36 μL of PBS (WT infection n = 18; *vacJ* infection n = 19); animals were monitored every 12 h. All animal studies were performed under protocols approved by the UCSD Institutional Animal Use and Care Committee with national and local guidelines in place to maximize humane animal treatment.

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CHAPTER 3

The Mla Pathway is Critical for *Pseudomonas aeruginosa* Resistance to Outer Membrane Permeabilization and Host Innate Immune Clearance

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Manuscript

Running title: Mla pathway in *Pseudomonas* Innate Immune Resistance

PREFACE TO CHAPTER 3

Chapter 3 is a manuscript being prepared for submission. The work in Chapter 3 addresses Aim 2a: Examine the role of VacJ/Mla pathway in *P. aeruginosa* as a potential therapeutic target. We investigated the innate immune resistance profile of a *P. aeruginosa vacJ* mutant and found it to be more susceptible to antimicrobial peptides, serum complement, and whole blood. Upon evaluation using an *in vivo* model of lung infection, a *P. aeruginosa vacJ* mutant showed reduced virulence potential when compared to wild-type parent strain. Chapter 3 establishes the importance of maintaining outer membrane lipid asymmetry, extending to additional Gram-negative pathogens. Our work also supports the notion that pharmacological targeting of the Mla pathway may be a broadly applicable therapeutic approach for all Gram-negative bacterial pathogens.

ABSTRACT

Pseudomonas aeruginosa is an important opportunistic pathogen that has become a serious problem due to increased rates of antibiotic resistance. Due to this along with a dearth in novel antibiotic development, especially against Gram-negative pathogens, new therapeutic strategies are needed to prevent a post-antibiotic era. Here we describe the importance of the vacJ/Mla pathway in resisting the bactericidal actions of the host innate immune response. *P. aeruginosa* tn5 transposon mutants lacking a functional VacJ/Mla pathway showed increased susceptibility to killing by the host cathelicidin antimicrobial peptide, LL-37 when compared to wild-type parent strain. *P. aeruginosa vacJ* mutant demonstrated increased membrane permeability upon damage autolysis in the presence of the detergent sodium dodecyl sulfate and the divalent cation chelator EDTA. When exposed to human whole blood and serum complement, *vacJ* mutant was killed more rapidly when compared to wild-type parent strain and complemented mutant. Finally, in an *in vivo* mouse lung infection model, infection with *vacJ* mutant resulted in reduced mortality, lower bacterial burden, and reduced lung damage when compared to wild-type strain. This study highlights the potential in therapeutically targeting the VacJ/Mla pathway in sensitizing *P. aeruginosa* to killing by the host innate immune response.

INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous Gram-negative facultative anaerobic bacterium that inhabits both soil and aqueous environments. A leading opportunistic human pathogen, *P. aeruginosa* is highly adaptable and intrinsically able to resist diverse antibiotic classes posing a tremendous challenge to medical management (1). *P. aeruginosa* preferentially colonizes immunocompromised patients thereby causing many nosocomial infections including ventilator-associated pneumonia, urinary tract infections, bacteremia, and infections of surgical sites or burn wounds (1, 2). Individuals with cystic fibrosis are at extreme risk of chronic pulmonary infection with *P. aeruginosa*, with the lungs of most patients colonized by age 3 (1). *P. aeruginosa* can also

cause community-acquired infections include keratitis, otitis externa, and skin and soft tissue infections. Therapeutic options are dwindling due to a decrease in novel antibiotic development as well as an emergence of highly multidrug-resistant *P. aeruginosa* strains, placing the pathogen on the Centers for Disease Control list of “serious threats” (3). The discovery of new targets and alternate strategies for treating *P. aeruginosa* infections is imperative.

Gram-negative bacteria such as *P. aeruginosa* are protected by their outer membrane (OM), which is highly impermeable to toxic compounds such as detergents, bile salts, and antibiotics (4). This OM is composed of an asymmetric bilayer containing lipopolysaccharide (LPS) on the outer leaflet and phospholipids (PLs) on the inner leaflet. Perturbation of the OM can lead to accumulation of PLs on the outer leaflet, which can make the bacteria vulnerable to further OM damage. The Mla system is involved in maintaining OM lipid asymmetry and is comprised of seven proteins that are distributed across the cell envelope (5, 6). VacJ (also known as MlaA) and other components of the Mla pathway have been shown to play a role in intracellular spread of *Shigella flexneri* (7, 8), for survival of *Haemophilus influenzae* during lung infections and in serum (9, 10), resistance to surfactants in *Escherichia coli* (6, 11, 12) and *Pseudomonas putida* (13), antibiotic resistance in *P. aeruginosa* (14, 15) and *Salmonella enterica* serovar Typhimurium (16), and has a possible role in the creation of outer membrane vesicles (17). VacJ has also been explored as a possible vaccine target in *Pasteurella multocida* (18). Genes encoding components of the Mla pathway including VacJ are upregulated in response to colistin and mutations in the pathway have been observed in colistin-resistant mutants (19, 20).

Among the critical frontline effectors of host innate immune defense are endogenous cationic antimicrobial peptides (AMPs), which are produced by epithelial cells and circulating or tissue-resident leukocytes, and are known to provide protection against invasive bacterial infections (21, 22). Cathelicidins are a family of mammalian AMPs with broad-spectrum antimicrobial activity against Gram-negative and Gram-positive bacteria, as well as certain fungi, viruses and protozoan parasites (23). The human cathelicidin AMP, LL-37, is an alpha-helical,

amphipathic and anionic peptide that integrates and destabilizes bacterial cell membranes causing their death (24). LL-37 also has important immunomodulatory roles in the proper regulation of cytokine release, chemotaxis, and antigen presentation (reviewed in (25)). Animals deficient in cathelicidin are hyper-susceptible to bacterial infection in multiple organs including skin (26), lung (27, 28), gut (29, 30), brain (31), kidney (32), and eye (33). A bacterial pathogen producing significant clinical disease in the human host can therefore be described as displaying some level of functional resistance to endogenous AMPs including cathelicidin in order for disease to persist (34, 35).

In this study, we explored for the first time the role(s) of the Mla pathway in *P. aeruginosa* resistance to host innate immune defenses and animal virulence. Though failing to show a growth defect in standard bacteriological and tissue culture media conditions, *P. aeruginosa* Mla pathway mutants showed increased susceptibility to human cathelicidin antimicrobial peptide LL-37 associated with increased membrane permeability. Compared to wild-type parent strain, an isogenic *P. aeruginosa vacJ* mutant showed increased susceptibility to LL-37 and the cationic peptide antibiotics colistin and polymyxin B, along with decreased survival in human whole blood and serum containing active complement; wild-type resistance to these factors was restored by complementation of the *vacJ* mutant with the wild-type gene with a transposon insertion. The *P. aeruginosa vacJ* mutant showed decreased virulence in a murine *in vivo* pneumonia model exhibited, as measured by lower bacterial burden in the lungs and blood, diminished lung injury and inflammation, and reduced mortality. Together, our results show the importance of the Mla pathway in *P. aeruginosa* resistance to the bactericidal activities of the innate immune system.

MATERIALS AND METHODS

Bacterial strains and reagents.

P. aeruginosa PAO1 (36) along with corresponding transposon Tn5 IS50L mutants (37) disrupting the genes encoding *mlaA* (*vacJ*) and *mlaC-F* were generously provided by Professor

Colin Manoil (University of Washington, Seattle, WA). Strains were propagated in Luria broth (LB) medium (Hardy Diagnostics) or on LB agar plates at 37 ºC. For infection studies, bacteria were grown to mid-log phase ($OD_{600\ nm}$ = 0.4) before diluting to experimental inocula. Complementation was performed as previously described (38). Briefly, *P. aeruginosa vacJ* gene and its upstream region was cloned into pUC18T-mini-Tn7T-*hph* using primers PA2800-miniTn7t-F (TGCTGCAAAGCCTGCACGGATCCACTAGTGAGCTCATGC), PA2800-miniTn7t-R (GAGGTGGAGGACGACTTCTAACCAGATAAGTGAAATCTAGTTCAAAAC), miniTn7t-PA2800-F (GCATGAGCTCACTAGTGGATCCGTGCAGGCTTTGCAGCA), and miniTn7T-PA2800-R (GTTTGGAACTAGATTTCACTTATCTGGTTAGAAGTCGTCCTCCACCTC). PA2800-miniTn7t-F and -R primers were used to amplify the pUC18T-mini-Tn7T-*hph* PCR product with portions of the *vacJ* gene, while primers miniTn7t-F and -R were used to amplify the *vacJ* gene with a portion of pUC18T-mini-Tn7T-*hph*. PCR products were combined 1:1 and electroporated into *E. coli* TOP10 cells, and transformants selected on LB agar plates containing 100 μg/mL Hygromycin B (Life Technologies).

Antibiotics and antimicrobial peptides.

For *in vitro* studies, colistin sulfate (Sigma-Aldrich) stock solutions were prepared in molecular grade water at 10,000 mg/L and frozen at -80 ºC. LL-37 and TAMRA fluorescently-tagged LL-37 were purchased from the American Peptide Company; stock solutions were prepared in molecular quality water at 640 μM and 320 μM, respectively, and stored at -80 °C. LL-37 minimum inhibitory concentration (MIC) assay were performed in RPMI containing 5% LB with 5 x 10^5 cfu/mL in 96-well round bottom plates (Corning).

NPN bacterial outer membrane permeability assay.

Assay was performed as previously described (39). Briefly, overnight cultures of *P. aeruginosa* were grown in LB at 37 °C while shaking, washed twice with PBS via centrifugation at

3,220 x g at room temperature, and resuspended to OD_{600nm} = 0.40 in 6 mL of RPMI + 5% LB. LL-37 was added to a final concentration = 4 μM to a subset of the bacterial cultures. Cultures were shaken at 37 °C for 1 h, spun at 3,000 x g at room temperature for 5 min, and resuspended in 0.5 mL of 10mM Tris buffer pH 8.0. The concentrated 0.5 mL cultures were used to prepare 1 mL bacterial stocks at OD_{600nm} = 0.40 in 10 mM Tris. Assays were conducted in a final volume of 200 μL in triplicate in 96-well flat bottom plates. Plates were immediately read in a fluorescent plate reader: excitation 250 nm & emission 420 nm.

Whole blood and serum killing.

Heparinized blood was collected from healthy volunteers and placed in siliconized tubes. 10^6 cfu of *P. aeruginosa* in RPMI was added so that the final concentration of whole blood was 90%. Samples were taken at time 0, 15, 30, 45, and 60 min, serially diluted, and plated on LB agar plates for cfu enumeration. Serum was collected from healthy volunteers by collecting blood in serum separating tubes (BD) which were spun at 1300 x g for 10 min to separate serum from erythrocytes and other cells. Collected serum was pooled and split; half was heat-inactivated at 56 ˚C for 30 min while the other half was stored at room temperature for 30 min. Serum was partitioned into wells of a 96 well round bottom plate and 1 x 10^6 cfu of *P. aeruginosa* wild-type, *vacJ*, or complemented mutant in RPMI was added to a final serum concentration of 80%. Samples were collected at 60, 120, and 180 min, serially diluted, and plated on LB agar for enumeration. Experiments were performed in triplicate.

Murine pneumonia model.

8-week-old female C57BL/6J mice (Jackson Labs) were used for. *P. aeruginosa* cultures were grown overnight in LB at 37 °C with shaking, washed twice with PBS, diluted 1:50 in fresh LB and grown to mid-log phase. Bacteria were washed twice with PBS via centrifugation at 3,220 x g at room temperature and concentrated in PBS to yield the appropriate inoculum. For

histopathology, lung bacterial load, and blood bacterial load, 4 x 10^6 cfu per 30 μL was used to infect each animal (WT infection n = 10; *vacJ* infection n = 10). Mice were anesthetized with 100 mg/kg ketamine + 10 mg/kg xylazine. Once sedated, the vocal chords were visualized using an operating otoscope (Welch Allyn) and 30 μL of bacteria or PBS instilled into the trachea during inspiration using a plastic gel loading pipette tip. Mice were placed on a warmed pad for recovery. After 24 h, mice were sacrificed with CO_2 and lungs were collected for bacterial counts and histopathological analysis. To enumerate bacteria remaining in the lungs, the organs were placed in a 2 mL sterile micro tube (Sarstedt) containing 1 mL of PBS and 1 mm silica beads (Biospec). Lungs were homogenized by shaking at 6,000 rpm for 30 sec (x3) using a MagNA Lyser (Roche), with the specimens placed on ice as harvested. Aliquots from each tube were serially diluted for enumeration on LB agar plates. For the *P. aeruginosa* survival experiment, 8.5 x 10^6 cfu were given in 30 μL of PBS (WT infection n = 15; *vacJ* infection n = 15); animals were monitored every 12 h for 7 d. All animal studies were performed under protocols approved by the UCSD Institutional Animal Use and Care Committee with national and local guidelines in place to maximize humane animal treatment.

RESULTS

Loss of function *mla* mutants in *P. aeruginosa* are hypersusceptible to antimicrobial peptides.

In recent years, the roles of the Mla pathway to resist stresses to the OM have been published; it is involved in resistance to hydrophobic compounds (13, 40), serum (10), and certain antibiotics (14). To determine if *P. aeruginosa* mutants containing transposon insertions in genes encoding Mla pathway proteins (**Fig 3.1A**) were susceptible to outer membrane attack by the human cathelicidin antimicrobial peptide LL-37, a minimum inhibitory concentration (MIC) assay was performed. Wild-type *P. aeruginosa* was able to resist the bactericidal action of LL-37 up to a concentration of 16 μM while all *mla* transposon insertion mutants revealed a 2-fold reduction in

MIC at 8 μM (**Fig 3.1B**). We next complemented one the *mla* mutants, *vacJ* with a mini-Tn7t transposon containing the *P. aeruginosa* PAO1 *vacJ* gene. The complemented *vacJ* mutant rescued the sensitizing effects revealed by an inactive Mla pathway back to wild-type resistance levels (**Fig 3.1B**). Challenging the *P. aeruginosa vacJ* mutant with the murine cathelicidin related antimicrobial peptide (mCRAMP) yielded a similar two-fold increased sensitivity compared to the wild-type and the *vacJ* complemented mutant strains (**Table 3.1**). Acceleration in LL-37 killing kinetics of *vacJ* mutant lacking a functional Mla system were observed in time course assays at 8 μM (**Fig 3.1C**) or 16 μM LL-37 (**Fig 3.1D**) compared to both the wild-type and the complemented strains.

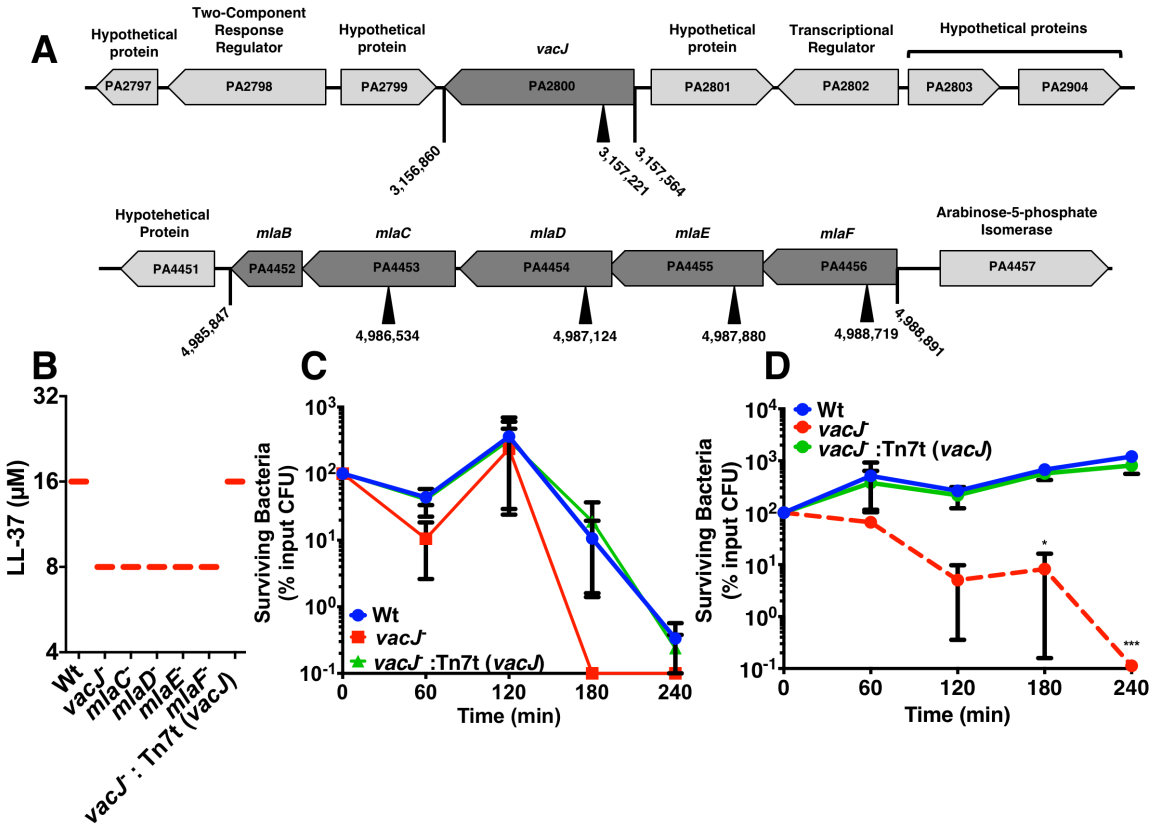


Figure 3.1 *P. aeruginosa mla* deficient mutants demonstrate increased sensitivity to antimicrobial peptide LL-37. Position of transposons for all *mla* deficient strains (A). LL-37 MIC for *P. aeruginosa* wild-type, *mla* deficient mutants, and *vacJ* complemented strains (B). LL-37 killing kinetics of *P. aeruginosa* WT, *vacJ*, and *vacJ* complemented strains at 16 μM (C) and 8 μM (D). * = $P < 0.05$, *** = $P < 0.001$ two-way ANOVA.

The related cationic peptide antibiotics colistin and polymyxin B are increasingly being used as last-line agents for treatment of patients with highly multidrug-resistant strains of *P. aeruginosa* (41, 42). Since these agents have similar charge characteristics and mechanisms of action to the host defense peptide LL-37, we examined the effect of the *vacJ* inactivation on sensitivity to these therapeutic agents. MIC testing revealed a 2-fold increase in susceptibility of the *vacJ* mutant *P. aeruginosa* to both polymyxin B and colistin, which was restored to wild-type resistance levels upon genetic complementation (**Table 3.1)**. These results show that the Mla pathway is needed in order to effectively resist the bactericidal effects of antimicrobial peptides that target the outer membrane.

Table 3.1 Summary of antimicrobial effects on *P. aeruginosa* wild-type, *vacJ* mutant, and complemented strain.

	***Pseudomonas aeruginosa* PAO1**		
Antimicrobial	**Wt**	***vacJ***	***vacJ*:*vacJ***
Cathelicidin LL-37	16 μM	8 μM	16 μM
mCRAMP	> 64 μM	32 μM	> 64 μM
Colistin	1 μg/mL	0.5 μg/mL	1 μg/mL
Polymyxin B	0.5 μg/mL	0.25 μg/mL	0.5 μg/mL

Analysis of *mla* deficient mutant susceptibilities to cell wall stress.

To ensure that cationic peptide hypersusceptibility of the *vacJ* mutant did not represent a general growth defect, we confirmed equal logarithmic growth properties throughout 24 h incubation in standard bacteriologic media compared to the WT parent strain (**Fig 3.2A**). To probe further into OM barrier function, we grew *P. aeruginosa* wild-type, *vacJ* mutant, and complemented strains on agar media containing 0.5% sodium dodecyl sulfate (SDS) with varying amounts of the divalent cation chelator ethylenediaminetetraacetic acid (EDTA), known to destabilize LPS bonds and increases OM permeability. We saw an increase in SDS-EDTA sensitivity of the *P. aeruginosa vacJ* mutant compared to the wild-type and complemented strains with increasing EDTA concentrations (**Fig 3.2B)**. To quantify the degree of permeability engendered, we performed an assay using the small molecule n-phenyl-1-napthylamine (NPN)

which fluoresces only when it is in a hydrophobic environment , in this case the inner membrane of Gram-negative bacteria, reflecting leakiness of the OM to allow small molecules to passively cross (43). No significant difference in NPN fluorescence was observed between *P. aeruginosa* wild-type and *vacJ* mutant at baseline (**Fig 3.2C**), but upon exposure to 4 μM LL-37, a 2-fold increase in permeability was seen in the *vacJ* mutant compared to wild-type. The complemented strain demonstrated reduced permeability to NPN at baseline and upon LL-37 treatment perhaps due to *vacJ* overexpression (**Fig 3.2C**). These results are consistent with a mechanism in which sensitivity to antimicrobial peptides can be attributed to an impaired OM repair in Mla pathway mutant.

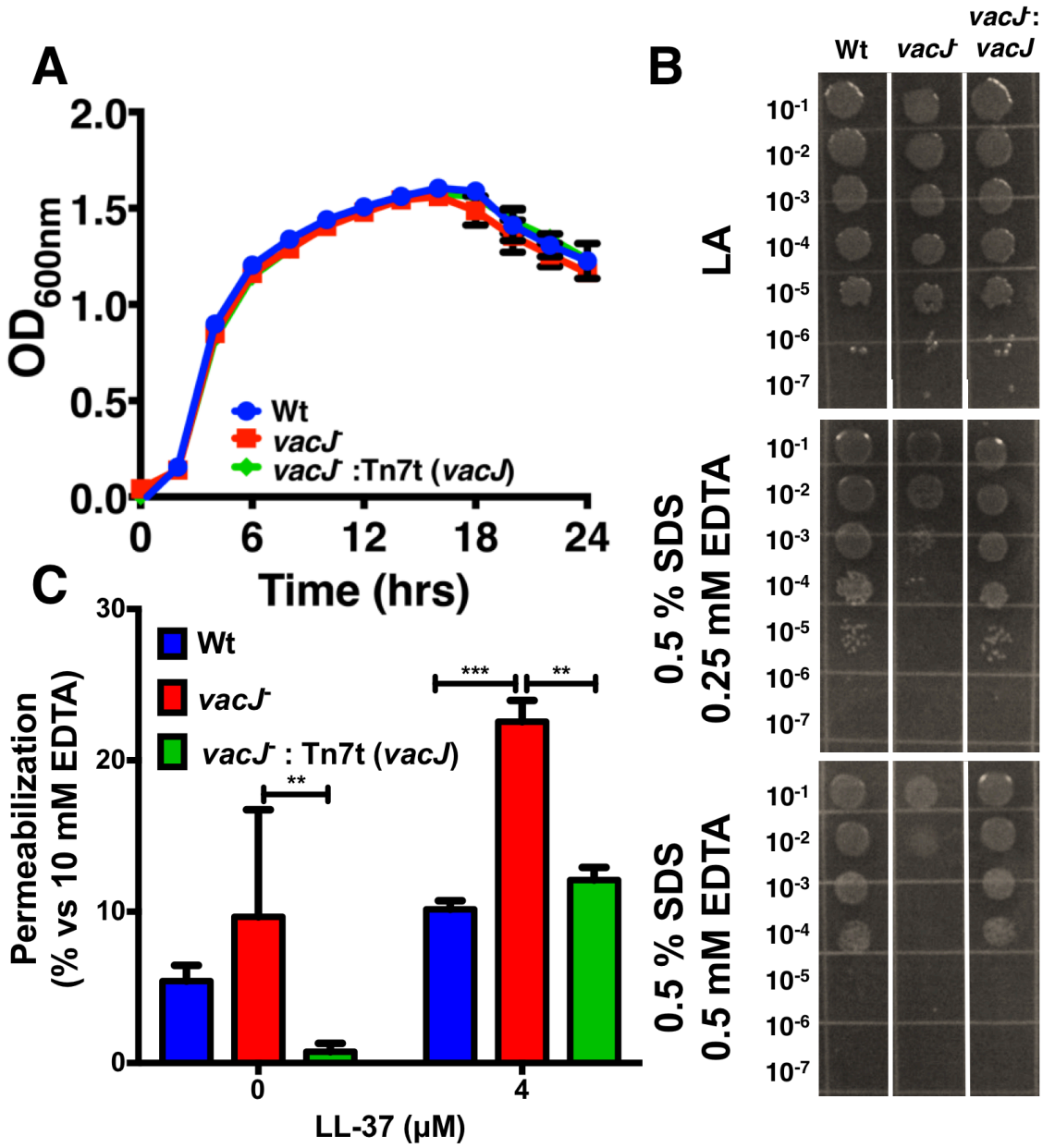


Figure 3.2 *P. aeruginosa mla* mutants are susceptible to OM stress with no defects in growth. Growth curves of *mla* mutants in Luria broth (A). Serial culture dilutions spotted on to solid media containing LB, LB + 0.5 % SDS and 0.25 mM EDTA, and LB + 0.5 % SDS and 0.5 mM EDTA (B). Representative graph of NPN fluorescent assay measuring outer membrane permeability in either RPMI + 5% LB or RPMI + 5% LB containing 4 μM LL-37 (C). ** = $P < 0.01$, *** = $P < 0.001$ two-way ANOVA.

***P. aeruginosa vacJ* mutant is susceptible to whole blood or serum killing**.

To further examine the *P. aeruginosa vacJ* mutant's ability to withstand outer membrane attack generated in the context of the innate immune system we used freshly isolated human whole blood that contains several OM active components (complement, platelet and neutrophil derived antimicrobial peptides and proteases, antibodies, etc.). Within 1h of incubation in human whole blood, we observe a log-fold increase in killing of the *vacJ* mutant compared to *P. aeruginosa* wild-type strain (**Fig 3.3A**); complementation of the mutant with the *vacJ* gene restored blood survival back to wild-type levels. Likewise, when placed in 80% human serum for 1 h, the *P. aeruginosa vacJ* mutant exhibited a nearly 2-logfold reduction in survival compared to wild-type and complemented mutant strains (**Fig 3.3B**). When the serum was heat-inactivated to degrade active complement, the survival differences between wild-type, *vacJ* mutant, and complemented mutant strain were lost. This finding illustrates the importance of having an intact Mla pathway to withstand serum- and complement-dependent killing in blood, consistent with experiments performed on the pathway in the Gram-negative coccobacillus *Haemophilus influenzae* (10).

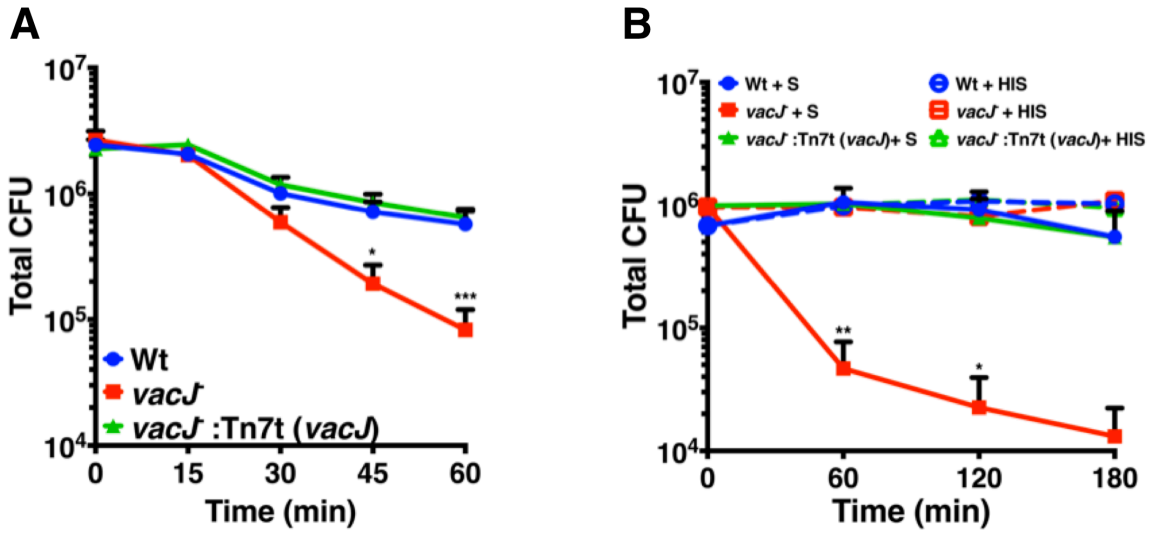


Figure 3.3 *Ex vivo* survival of *P. aeruginosa mla* defective mutant *vacJ*. 2.5 x 10^6 bacterial cfu in RPMI was placed in heparinized human whole blood to make final concentration 90% blood in final volume of 200 μL (A). 1 x 10^6 bacterial cfu in RPMI was placed in either serum or heat inactivated serum so final concentration was 80% serum in final volume of 200 μL (B). * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ two-way ANOVA.

***P. aeruginosa* Mla pathway is essential for full version in murine pneumonia model**.

To determine the essentiality of the Mla pathway during *P. aeruginosa* lung infections, we used a murine pneumonia model in which 8.5 x 10^6 cfu of either *P. aeruginosa* wild-type or *vacJ* mutant were administered intratracheally to 8-week-old female C57BL/6 mice. Mouse survival, bacterial load, bacteremia, and histopathology of the lungs of infected mice were assessed. *P. aeruginosa* wild-type infection led to 100% mortality within 3 d while the *vacJ* infected mice had 40% survival at the same time point and a final survival of 27% at the end of the 7 d experiment ($P < 0.05$) (**Fig 3.4A**). In a separate cohort of mice, we harvested and homogenized lungs to determine the bacterial burden 24 h post-infection (hpi) and found a 2-fold reduction in *vacJ* mutant bacterial recovery compared to a wild-type infection ($p < 0.05$) (**Fig 3.4B**). At this time point, we also observed a 3-fold reduction in bacterial cfu in the blood of mice infected with *P. aeruginosa vacJ* mutant compared to wild-type infection; 4 of the 6 *vacJ* infected mice had bacterial counts below 1x10^6 cfu/mL while 5 of 6 wild-type infected mice had bacterial counts

greater than 1x10^6 cfu/mL ($P < 0.05$) (**Fig 3.4C**). Examination of histopathology performed on lung sections from mice infected with either *P. aeruginosa* wild-type or *vacJ* mutant revealed differences in the severity of lung injury (**Fig 3.4D**). Wild-type infected mice showed increased neutrophil infiltration and damage to the alveolar architecture compared to *vacJ* mutant infection, where histopathological changes were significantly diminished and focal points of neutrophil infiltration were fewer and smaller. These findings demonstrate enhanced pulmonary innate immune clearance of the *P. aeruginosa vacJ* mutant in concert with decreased propensity to bloodstream dissemination.

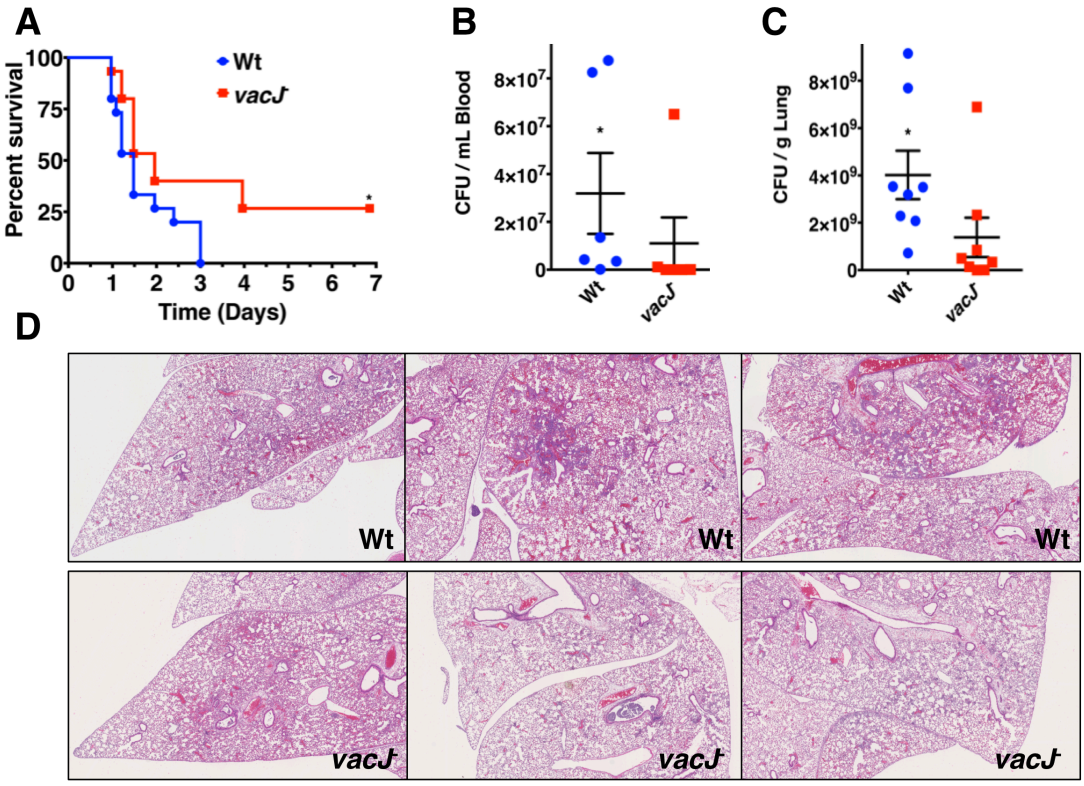


Figure 3.4 Effect of *vacJ* mutation on *P. aeruginosa* virulence in murine pneumonia model. Mice (n = 15 per group) were infected with *P. aeruginosa* wild-type or *vacJ* (8.5 x 10^6 cfu / 30 μL PBS) intratracheally and observed for survival over a period of 7 d; * = $P < 0.05$ Log-rank (Mantel-Cox) test (A). Mice (n = 8 per group) were infected with *P. aeruginosa* wild-type or *vacJ* (4 x 10^6 cfu/ 30 μL) intratracheally and sacrificed 24 h post infection. Lungs were harvested (B) and blood collected (C) for bacterial enumeration. * = $P < 0.05$ two-tailed Mann-Whitney test. (D) Histology of fixed lung sections from mice infected with either wild-type or *vacJ* mutant and stained with haemotoxylin and eosin.

DISCUSSION

Nosocomial bacterial infections have become an ever-increasing public health concern, with 650,000 individuals experiencing health care-associated infections per year, at least 20% of which are characterized by antibiotic resistance (44). Sustained selective pressure caused by the constant use of antibiotics has allowed the rise of a select group of pathogens commonly isolated multidrug-resistant profiles (45). The most challenging threats in the so-called “ESKAPE group” of pathogens are the Gram-negatives due to the dearth of novel antibiotic candidates in the pipeline to treat these infections (46). Among Gram-negative bacterial threats, *Pseudomonas* is of particular concern due to ubiquitous nature of the pathogen and its intrinsic ability to resist many antibiotic classes and mechanisms of action. *P. aeruginosa* is a recurring problem in nearly all cystic fibrosis (CF) patients beginning in early childhood (1) and increasing rates of *P. aeruginosa* antibiotic resistance have been recently observed in isolates from central line-associated bloodstream infection as well as ventilator-associated pneumonia (44).

The Mla pathway plays a critical role in maintaining OM integrity in different Gram-negative infections, but has not been studied in great detail in the context of infection and innate immune resistance among the leading antibiotic-resistant human pathogens. Here we report that *P. aeruginosa* deficient in the Mla pathway and the gene *vacJ* in particular, though exhibiting normal growth characteristics *in vitro*, demonstrate increased sensitivity to endogenous and pharmaceutical cationic antimicrobial peptides, serum, and whole blood. A *vacJ* mutant also showed decreased virulence in a murine model of *P. aeruginosa* lung infection, with reduced bacterial burden and increased survival compared to wild-type infected animals.

With the rapid rate of emerging Gram-negative bacterial resistance to modern antibiotics, it is critical to continue to explore alternative strategies and drug targets to combat these pathogens. Molecules designed to disrupt Gram-negative OM stability are being investigated, and

show great promise as a therapeutic strategy (47). In particular, targeting the Mla pathway could be an interesting pharmacological target as it is conserved amongst all Gram-negative bacteria (17) and therefore offers the potential for broad-spectrum activity and clinical utility. Our research suggests that inhibitors of the Mla pathway in *P. aeruginosa* would not be directly bactericidal, but would rather sensitize Gram-negative bacteria to killing by the host's endogenous defenses (e.g. antimicrobial peptides, serum complement), potentially limiting collateral damage to the host microbiome that can be seen with repeated broad-spectrum antibiotic administration. The Mla system has also been reported to be important in maintaining colistin resistance in *A. baumannii* (19, 48); inhibitors may be able to reverse colistin resistance and render them susceptible to a last line of defense antibiotic used for Gram-negative infections.

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CHAPTER 4

Azithromycin Synergizes with Cationic Antimicrobial Peptides to Exert Bactericidal and Therapeutic Activity Against Highly Multidrug-Resistant Gram-Negative Bacterial Pathogens

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PREFACE TO CHAPTER 4

Chapter 4 in full is a published journal article in *EBioMedicine* in which I was a co-author. My work helps address Aim 2b: Explore how the *in vivo* permeabilization of the Gram-negative outer membrane by host cationic antimicrobial peptides reveals potent bioactivities of azithromycin overlooked in standard antimicrobial testing. This was done by developing the *Acinetobacter baumannii* lung infection model to test if the innate immune response sensitized bacteria to the macrolide antibiotic, azithromycin. This experiment revealed that in the presence of an active innate immune response which contains many membrane active components, such as complement and antimicrobial peptides, damage to the outer membrane was enough to allow azithromycin to penetrate into the bacterial cell therefore synergizing with natural antibiotic defenses.

ABSTRACT

Antibiotic resistance poses an increasingly grave threat to the public health. Of pressing concern, rapid spread of carbapenem-resistance among multidrug-resistant (MDR) Gram-negative rods (GNR) is associated with few treatment options and high mortality rates. Current antibiotic susceptibility testing guiding patient management is performed in a standardized manner, identifying minimum inhibitory concentrations (MIC) in bacteriologic media, but ignoring host immune factors. Lacking activity in standard MIC testing, azithromycin (AZM), the most commonly prescribed antibiotic in the U.S., is never recommended for MDR GNR infection. Here we report a potent bactericidal action of AZM against MDR carbapenem-resistant isolates of *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, and *Acinetobacter baumannii*. This pharmaceutical activity is associated with enhanced AZM cell penetration in eukaryotic tissue culture media and striking multi-log-fold synergies with host cathelicidin antimicrobial peptide LL-37 or the last line antibiotic colistin. Finally, AZM monotherapy exerts clear therapeutic effects in murine models of MDR GNR infection. Our results suggest that AZM, currently ignored as a treatment option, could benefit patients with MDR GNR infections, especially in combination with colistin.

INTRODUCTION

Hospital-acquired infections, half caused by drug-resistant bacteria (1), cause ~99,000 deaths annually and increase healthcare costs by $5-10 billion in the U.S. alone (2). Recent reports by the U.S. Centers for Disease Control and Prevention (3) and the World Health Organization (4) describe this ever-worsening antibiotic resistance crisis, highlighting the "urgent threat" of emerging carbapenem-resistant Gram-negative rods (GNRs) that forebode the entry of human medicine into a "post-antibiotic era". Rapid spread of carbapenem resistance in *Pseudomonas aeruginosa* (*PA*), *Klebsiella pneumoniae* (*KP*) and *Acinetobacter baumannii* (*AB*) is of particular concern as effective antibiotic candidates are currently lacking in the development pipeline (5).

Unconventional approaches to infectious disease treatment are gaining more attention, including virulence factor inhibition, bacteriophage therapy, probiotics and immune boosting (6, 7). Along these lines, we have probed interactions of conventional antibiotics with antimicrobial effectors of the innate immune system, with encouraging results. Drugs with no direct activity in standard minimum inhibitory concentration (MIC) testing nevertheless sensitized multidrug-resistant (MDR) bacterial pathogens to human antimicrobial peptide killing *in vitro*, e.g. nafcillin vs. methicillin-resistant *Staphylococcus aureus* (MRSA) or ampicillin vs. vancomycin-resistant *Enterococcus* (VRE), and contributed to clinical resolution in refractory infections (8, 9).

Our recent experiences with β-lactams and MRSA or VRE indicate that simple MIC testing overlooks potential synergies with cationic antibiotics (e.g. daptomycin) and host AMPs (e.g. human cathelicidin LL-37) that promote bactericidal activity *in vitro* and bacterial clearance in patients (8, 9). We asked whether similar phenomena could be identified in MDR-GNRs to challenge conventional antibiotic treatment paradigms. AZM, the most commonly prescribed antibiotic in the U.S. (51.5 million in 2010) (10), is never recommended for inpatient treatment of serious GNR infections because of poor or absent *in vitro* activity by standard MIC testing in bacteriologic media. However, antibacterial activity of AZM is enhanced in mammalian tissue

culture media vs. standard bacteriologic media (11), a finding reminiscent of observations we made for LL-37 (12), prompting us to examine its interaction with MDR GNRs more closely.

MATERIALS AND METHODS

2.1. Bacterial Strains

P. aeruginosa (*PA*) strain PA01, *K. pneumoniae* (*KP*) strain K700603, and *A. baumannii* (*AB*) strain AB19606 were obtained from the American Type Culture Collection (ATCC). Human clinical MDR isolates *PA* P4 (lung) and *KP* K1100 (lung) (13) were obtained from a tertiary academic hospital in the New York metropolitan area. MDR-*AB* AB5075 (bone) (14) was obtained from Walter Reed Army Medical Center. All three MDR strains were independently identified and subject to antibiotic susceptibility testing by the clinical microbiology laboratory at the San Diego Veterans Affairs Hospital (**Table S1**). Additional MDR GNR clinical isolates tested were also obtained from a tertiary academic hospital in the New York metropolitan area, except for PA USCD P1, which was obtained from the UC San Diego hospital system. Bacteria were grown overnight in Luria Broth (LB), glycerol was added (30% final), and stocks stored at -80^{0}C. Fresh colonies were streaked onto LB plates each week for all experiments.

2.2. Antibiotics and Antimicrobial Peptides

For *in vitro* studies, AZM, colistin sulfate, and ciprofloxacin were purchased from Sigma-Aldrich; erythromycin and clarithromycin were purchased from Fischer Scientific. Stock solutions were prepared in phosphate buffered saline (PBS) at 2,560 mg/L for the macrolide antibiotics, 1,000 mg/L for colistin, and 10,000 mg/L for ciprofloxacin. Trace amounts of glacial acetic acid were used to prepare AZM, erythromycin and clarithromycin stocks for complete solubility (15). LL-37 and TAMRA-tagged LL-37 were purchased from the American Peptide Company; stock solutions were prepared in molecular quality water (Corning Cellgro) at 640 μM and 320 μM respectively and stored at -80^{0}C. For *in vivo* studies, AZM for human injection (Sagent Pharmaceuticals) was reconstituted per manufacturer's guidelines (16). Pooled human serum

was obtained from six healthy consented lab volunteers and immediately aliquoted and stored at -80^{o}C.

2.3. Reagents

Mueller-Hinton Broth (MHB, Spectrum Chemicals) was supplemented with $CaCl_2$ and $MgCl_2$ to make cation-adjusted MHB (Ca-MHB) – final cation concentrations (20–25 mg/L Ca^{2+} and 10–12.5 mg/L Mg^{2+}). Luria Broth base (LB) was purchased from Hardy Diagnostics. RPMI-1640 was purchased from Invitrogen. Clear phenol free RPMI-1640 used for microscopy studies was purchased from Corning Cellgro. 1-N-phenylnaphthylamine (NPN) was purchased from Sigma-Aldrich and a fresh stock of 500 μM in acetone was made each week.

2.4 MIC Determinations

MIC values for AZM, erythromycin, clarithromycin, ciprofloxacin, colistin, and LL-37 were determined using broth microdilution in accordance with the Clinical Laboratory Standards Institute (CLSI) guidelines (17) using Ca-MHB media, the recommended bacteriologic broth, or eukaryotic cell culture media RPMI-1640 supplemented with 5% LB (9).

2.5. Time-Kill Curves and Serum Survival Assays

Time-kill studies, $\pm$ 20% pooled human serum, were performed as previously described (18). Bacteria were grown overnight in LB at 37^{o}C with shaking. Bacterial stocks in PBS were prepared by washing the overnight cultures twice with PBS via centrifugation at 3220 x *g* at room temperature with a final re-suspension in PBS to an OD_{600} = 0.40. Bacterial stocks in PBS were diluted in Ca-MHB or 5%LB-RPMI to an initial inoculum of $1x10^6$ CFU/ml (standard time-kill) or $5x10^4$ CFU/ml (serum survival). AZM, erythromycin, clarithromycin, colistin, and LL-37 stocks were diluted in Ca-MHB or 5% LB-RPMI to the assay concentrations indicated. For serum studies, AZM 0.5 mg/L was chosen in order to approximate human plasma concentrations upon intravenous administration of 500mg of AZM (16). Assays were conducted in triplicate in a final volume of 200µl in 96-well round bottom plates (Costar) $\pm$ 20% pooled human serum. The 96-well plates were wrapped in paraffin and placed in a shaking incubator at 37^{o}C. Aliquots were

collected at the indicated times and serially diluted for CFU enumeration; limit of detection = CFU/ml.

2.6. Electron Microscopy of Bacterial Gross Morphology

Transmission electron microscopy was performed essentially as described (19). MDR-*AB* was grown overnight in LB at 37^{o}C with shaking. Bacterial stocks in PBS were prepared by washing the overnight cultures twice with PBS and resuspending in PBS to OD_{600} = 0.40. Then 2.5 ml of each bacterial stock was added to 47.5 ml of Ca-MHB or RPMI + 5% LB media pre-warmed to 37^{o}C. For treatment, the AZM stock was diluted to a concentration of 0.5 mg/L in the final volume of 50 ml. 50 ml cultures were placed in a shaking incubator at 37^{o}C for 2 h. Cultures were then centrifuged at 3220 x g at room temperature for 10 min. The supernatant was aspirated and bacterial pellets re-suspended in 1 ml of PBS. These 1ml samples were immersed in modified Karnovsky's fixative (2.5% glutaraldehyde and 2% paraformaldehyde in 0.15 M sodium cacodylate buffer, pH 7.4) for at least 4 h, post-fixed in 1% osmium tetroxide in 0.15 M cacodylate buffer for 1 h, and stained en bloc in 2% uranyl acetate for 1 h. Samples were dehydrated in ethanol, embedded in Durcupan epoxy resin (Sigma-Aldrich), sectioned at 50-60 nm on a Leica UCT ultramicrotome, and picked up on Formvar and carbon-coated copper grids. Sections were stained with 2% uranyl acetate for 5 min and Sato's lead stain for 1 min. Grids were viewed using a Tecnai G2 Spirit BioTWIN transmission electron microscope and photographs were taken with an Eagle 4k HS digital camera (FEI). Images were taken from multiple random fields at 1,200X, 2,900X, 23,000X; and gross morphology was analyzed in a blinded fashion.

2.7. Fluorescence Microscopy for MDR-AB Cytological Profiling

The fluorescence microscopy studies for MDR-*AB* were performed as previously described (20). These microscopy studies required higher concentrations of AZM because the concentration of MDR-*AB* used was 100X higher (5 x 10^{7} CFU/ml) compared to the concentration used in the MIC assays (5x10^{5} CFU/ml). All AZM concentrations used in these studies are pharmacologically obtainable in human tissue. Single MDR-*AB* colonies were picked from LB

plates and grown in LB or RPMI + 5% LB overnight. Overnight cultures where then diluted 1:100 into fresh Ca-MHB or RPMI + 5% LB media. When an OD_{600} = 0.20 was reached, antibiotics were added to exponentially growing bacteria. Cultures were placed on a shaker at 30^{o}C and collected after 1 h or 2 h and stained with 1 µg/ml FM4-64 2 µg/ml DAPI, and 0.5 µM SYTOX-Green (Molecular Probes/Invitrogen). In the case of NBD-tagged AZM, SYTOX-Green dye was omitted and cell cultures were washed with fresh media before staining. Stained cultures were centrifuged at 300 x *g* for 30 sec in microcentrifuge and resuspended in approximately 5% original volume. 3 µl of concentrated cells were transferred onto a pad containing 1.2% agarose and 20% LB medium for microscopy. The exposure time of each excitation was the same for each experimental replicate included in the statistical analysis of all treatments. All images were analyzed using ImageJ software v1.48f and CellProfiler 2.0. Fluorescence intensity analysis was performed on non-deconvolved images. Average DAPI, SYTOX Green, or NBD intensity per cell was measured and respective background intensities subtracted. Finally, the intensity of treated cells was normalized by the intensity of untreated cells from the same experiment set, making intensity data from different experimental sets comparable as relative intensity. Protein translation inhibition phenotype was automatically calculated by the degree of DNA condensation defined by the ratio of DNA area over total cell membrane area. Cells with a ratio less than half of those calculated from untreated control cells were considered to have condensed toroid nucleoids (20). Fluorescence intensity and protein translation inhibition population data were obtained by counting >500 cells from random microscopy fields per condition per experimental replicate, with 3 independent replicates. The fluorescence microscopy for LL-37 cell binding was performed in a similar manner except that after the MDR-*AB* cultures were incubated in 0.5 mg/L of AZM versus control for 2 h, 2 µM of TAMRA-tagged LL-37 was added and the cultures incubated for an additional 30 min before staining and analysis.

2.8. Mouse AZM Dosing

Standard human dosing of AZM is 500 or 1,000 mg administered orally or intravenously q 24 h (7.7 or 15 mg/kg for an average adult). Mice metabolize AZM 29 times faster than humans (21), and 50 mg/kg AZM given subcutaneously to mice approximates 500 mg given intravenously to human patients (22). We therefore chose to administer 50 or 100 mg/kg AZM subcutaneously in our murine infection models once every 24 h.

2.9. Mouse Pneumonia Models

The murine pneumonia mode was performed with slight modifications as previously described (23). All intratracheal infections were performed in a blinded fashion with respect to AZM or PBS treatment. For the MDR-*AB* lung infection model, 8-wk-old female C57Bl/6J mice (Jackson Labs) were used. MDR-*AB* cultures were grown overnight in LB at 37^{o}C with shaking and then re-grown in the morning in fresh LB to a concentration of OD_{600} = 0.40. Bacteria were washed twice with PBS via centrifugation at 3220 x *g* at room temperature and concentrated in PBS to yield 5x10^{6} CFU in the 30 μl, the inoculation volume. Mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine. Once sedated, the vocal chords were visualized using an operating otoscope (Welch Allyn) and 30 μl of bacteria or PBS was instilled into the trachea during inspiration using a plastic gel loading pipette tip. Mice were placed on a warmed pad for recovery and given one subcutaneous dose of 50 mg/kg or 100 mg/kg human AZM for injection reconstituted in PBS. Mice were sacrificed with CO_2 for bacterial counts or for analysis of their broncho alveolar fluid (BALF) 24 h after infection. To enumerate total surviving bacteria in the lungs, both lung lobes were removed and placed in a 2 ml sterile micro tube (Sarstedt) containing 1 ml of PBS and 1mm silica beads (Biospec). Lungs were homogenized by shaking twice at 6,000 rpm for 1 min using a MagNA Lyser (Roche), with the specimens placed on ice as soon as they were harvested. Aliquots from each tube were serially diluted for CFU enumeration on LB plates. For BALF collection and analysis, an incision was made in the trachea and the lungs slowly inflated with 700 μl of chilled PBS using a 1 ml syringe topped with a 20G blunt needle tip (Harvard Apparatus). The PBS was slowly withdrawn, and the recovered BALF was spun at 1200

x *g* at 4°C for 10 min. The supernatant (350 *µ*l) was analyzed for the mouse pro-inflammatory cytokines mIL-1β, mIL-6, and mMIP-2 (R&D Systems ELISA Kits). Cell pellets were resuspended in chilled PBS to a final volume of 500 µl. Samples were enumerated with a hemocytometer for total leukocyte number. 100 µl was spun onto glass microscopy slides using a Shandon Cytospin 3 (Thermo Scientific). Dry glass slides were stained with Wright-Giemsa stain, and >200 leukocytes were counted per animal using a light microscope for neutrophil and alveolar macrophage enumeration. For the MDR-*AB* lung infection survival experiment $5x10^7$ CFU were given in 36 µl of PBS. One dose of 100 mg/kg AZM or 100 µl of PBS control was given subcutaneously immediately after the infection (time 0) and a second dose was given 24 h later. The MDR *KP* and *PA* intra-tracheal murine lung infections were performed in a similar manner except that 8-week-old female CD1 mice (Charles River Labs) were used and the animals were sacrificed 36 h after infection for enumeration of surviving bacteria. The inoculums for MDR-*KP* and MDR-PA infection were $1.5x10^7$ CFU and $1x10^7$ CFU in 40 *µ*l of PBS, respectively. The *AB* foreign body infection studies were performed as previously described (24). All animal studies were performed under protocols approved by the UCSD Institutional Animal Use and Care Committee.

2.10. Statistical Analysis

All statistics were performed using GraphPad Prism version 5.0 2-way ANOVA, 1-way ANOVA, two tailed student's t-test, and log-rank test were performed as described in each figure legend.

RESULTS

3.1. AZM is Highly Bactericidal vs. MDR GNRs in Tissue Culture Media

For each opportunistic GNR pathogen (*PA*, *KP and AP*) we tested a common model strain plus a corresponding extremely MDR human isolate (**Table S 4.1**). AZM MICs were determined by Clinical and Laboratory Standard Institute (CLSI) broth microdilution methodology (17) using either the recommended cation-adjusted Mueller-Hinton broth (Ca-MHB) or eukaryotic

cell culture media RPMI-1640 supplemented with 5% Luria broth (RPMI + 5% LB) (**Table S 4.2**). In every case, a ≥30-fold reduction in MIC was seen in RPMI + 5% LB vs. Ca-MHB, rendering all organisms susceptible by the accepted breakpoint of ≤8 mg/L for *Campylobacter* and *S. aureus*. In contrast, no marked changes in MIC of fluoroquinolone ciprofloxacin were observed for model strains (all sensitive) or MDR clinical isolates (all resistant) in RPMI + 5% LB vs. Ca-MHB (**Table S 4.2**). A similarly profound reduction in MIC in RPMI + 5% LB vs. Ca-MHB was also observed for an expanded panel of 11 additional MDR GNR clinical isolates (**Table S 4.3**). Two additional macrolides, erythromycin and clarithromycin, also demonstrated dramatically higher activity against MDR GNRs in RPMI + 5 % LB versus Ca-MHB, although AZM was the most potent of the macrolides tested (**Table S 4.2**). Time-kill curves determined the mean bactericidal concentration (MBC, reduction in CFU by 2 $\log_{10}$) of AZM against the MDR-GNRs in RPMI + 5% LB media (**Fig 4.1A-C**). With an initial inoculum of $1x10^6$ CFU/ml, the MBC of AZM against MDR-*PA, -KP* and -*AB* were 4 mg/L (MIC = 2 mg/L), 1 mg/L (MIC = 1 mg/L) and 0.5 mg/L (MIC = 0.5 mg/L) respectively. While all three MDR-GNRs achieved rapid logarithmic growth in both RPMI + 5% LB and Ca-MHB, AZM was markedly more bactericidal in the eukaryotic tissue culture media (**Fig 4.1A-C**): a striking 6-logfold increased killing for *KP* and *AB* and 2-logfold increased killing for *PA*. For MDR-*PA* and –*AB*, AZM 4 mg/L (<1/16 MIC) resulted in significant killing even in Ca-MHB once bacteria reached stationary growth phase (**Fig 4.1A and C**), consistent with reported AZM bactericidal activity against stationary phase antibiotic-sensitive *PA* (25). AZM activity against all three MDR pathogens was further enhanced in the presence of 20% human serum (**Fig 4.1D-F**), mirroring a prior observation of serum potentiation of AZM activity against *E. coli* and *S. aureus* (26). Serial passage of all three MDR pathogens for 10 consecutive days at sub-minimum inhibitory concentrations of AZM in RPMI + 5% LB media demonstrated no increase in resistance to AZM over this time frame (**Fig S 4.1**).

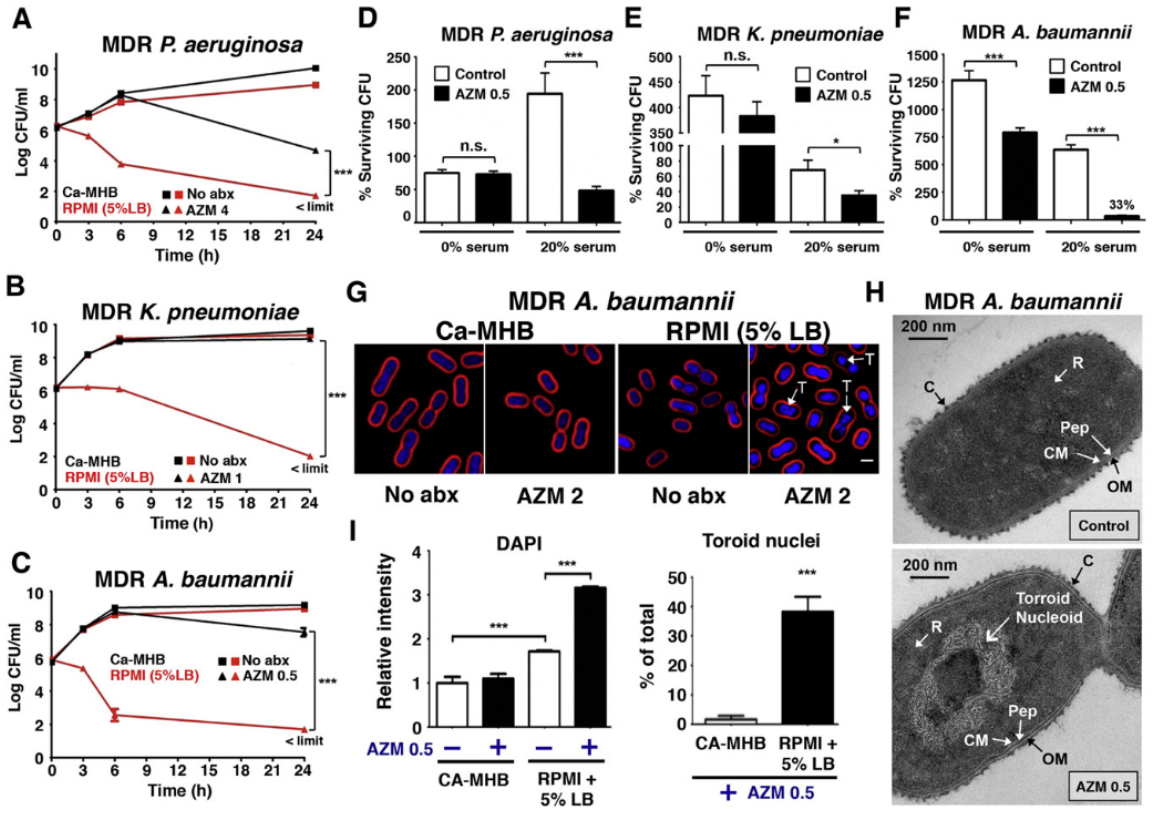


Figure 4.1 AZM bactericidal activity against GNRs in eukaryotic media and human serum. AZM concentrations = mg/L. (A-C) Time-kill curves demonstrating AZM MBC against MDR *PA*, *KP*, and *AB* in eukaryotic media (RPMI + 5% LB) vs. bacteriologic broth (Ca-MHB). Mean of triplicates from 3 independent experiments ± SEM ***$P < 0.001$; two-way ANOVA; limit of detection 50 CFU/ml. (D-F) $5x10^4$ CFU/ml bacteria incubated at 37°C for 2 h in RPMI alone (0% serum), AZM 0.5, 20% serum, and AZM 0.5 + 20% serum. Data show % viable CFU vs. initial inoculum; mean of triplicates from 3 independent experiments ± SEM. *$P < 0.05$, ***$P < 0.001$; two-tailed Student's t-test. (G) MDR *AB* ($5x10^7$ CFU/ml) grown in Ca-MHB vs. RPMI + 5% LB were treated for 2 h with AZM 2 and stained for fluorescence microscopy: FM4-64 (red cell membrane stain), DAPI (blue DNA stain), "T" denotes toroid shaped nucleoid. For ready visualization, the concentration of MDR *AB* was 100-fold higher than in MIC assays; thus a higher concentration AZM was used. (H) Transmission electron microscopy images representative of 2 independent experiments with logarithmic growth phase MDR *AB* treated for 2 h with AZM 0.5; C = Capsule; CM = Cell Membrane; Pep = Peptidoglycan; OM = Outer Membrane; R = Ribosomes. (I) Bar graphs generated from unbiased software analysis of multiple random microscopy fields with > 500 cells counted per condition per experimental replicate. Data representative of 3 independent experiments plotted as mean ± SEM; additional microscopy details in methods section. ***$P < 0.001$; one-way ANOVA and two tailed student's t-test.

3.2. Enhanced AZM Permeability in Tissue Culture Media Triggers Nucleoid Collapse

AZM binds the 50S large ribosomal subunit at the polypeptide exit tunnel, blocking protein synthesis (27). We employed fluorescence microscopy-based bacterial cytological profiling (BCP) (20) of MDR-*AB* to identify the cellular pathway perturbed by AZM treatment in RPMI + 5% LB media but not Ca-MHB. BCP revealed that nucleoid collapse into a low energy toroid shape, a hallmark of protein synthesis inhibition (20, 28), was markedly increased in *AB* treated with AZM in RPMI + 5% LB (**Fig 4.1G**), a finding corroborated by transmission electron microscopy of treated cells (**Fig 4.1H**). Increased staining intensity of the DNA dye DAPI in RPMI + 5% LB vs. Ca-MHB even without AZM suggested increased membrane permeability in the cell culture media (**Fig 4.1I**). DAPI staining of MDR-*AB* was further increased upon AZM treatment, possibly reflecting impaired efflux pump function seen in antibiotic-sensitive *PA* treated with AZM in RPMI (11).

3.3. Colistin Permeabilization Markedly Potentiates AZM Bactericidal Activity vs. MDR GNRs

Colistin is an antibiotic of last resort for carbapenem-resistant GNR infections (29), but its use is complicated by marked dose-dependent nephrotoxicity and uncertainties in optimal dosing (30). We asked whether AZM could act synergistically with colistin, even in standard Ca-MHB media in which AZM alone has little or no activity. At a sub-MIC (**Table S 4.2**) and pharmacologically attainable dose of each drug, marked synergy (31) of AZM + colistin was observed against MDR-*PA* (additional reduction in CFU by 2 $\log_{10}$), -*KP* (by 5 $\log_{10}$) and -*AB* (by 3 $\log_{10}$) (**Fig 4.2A-C**). BCP showed strongly increased DAPI staining and toroid nucleoid morphology in MDR-*AB* treated with AZM + colistin vs. either agent alone (**Fig 4.2D and E**). A membrane permeability effect was corroborated as bacterial cell entry of the fluorescent dye SYTOX green (MW = 600 Da vs. 749 Da for AZM) was increased 3-fold by colistin and 4-fold by colistin + AZM (**Fig 4.2D and G**). Indeed, colistin markedly enhanced entry of fluorescently tagged AZM (NBD-AZM (32)) into MDR-*AB* cells (**Fig 4.2F and G**). A cationic peptide antibiotic,

colistin permeabilizes the bacterial cell envelope (33), and we surmise it facilitates AZM entry whereupon the latter drug can exert its classical ribosomal protein synthesis activity. Erythromycin and clarithromycin also showed significant synergy with colistin (**Fig S 4.2**), with azithromycin the most potent of the three macrolides tested.

3.4. Pronounced Bactericidal Synergy of AZM with Human Cathelicidin LL-37 vs. MDR GNRs

Endogenous cationic AMPs are critical to mammalian innate immunity against invasive bacterial infection(34). Given the observed synergy of AZM with the bacterial-derived AMP colistin, we hypothesized that AZM could be potentiated by LL-37, a cationic AMP produced abundantly by phagocytic and epithelial cells during infection (35). At sub-MIC doses (**Table S 4.2**), marked synergy of AZM + LL-37 was observed against MDR-*PA* (additional reduction in CFU by 4 $\log_{10}$), *KP* (7 $\log_{10}$) and *AB* (4 $\log_{10}$) (**Fig 4.3A-C**). For MDR-*AB*, AZM 0.0625 mg/L ($1/8^{th}$ MIC) + 1μM LL-37 ($1/4^{th}$ MIC) resulted in >99% killing within 4 h. BCP showed increased DAPI staining and toroid morphology of nucleoids in MDR-*AB* treated with AZM + LL-37 vs. either agent alone (**Fig 4.3D and E**). SYTOX green entry into the bacterial cell was increased 3-fold in the presence of LL-37 and 5-fold in the presence of LL-37 + AZM (**Fig 4.3D and G**). LL-37 also markedly enhanced entry of NBD-AZM into MDR-*AB* cells (**Fig 4.3F and G**), consistent with the known activity of LL-37 to interfere with GNR cell wall biogenesis and cytoplasmic membrane integrity (36). Studies with 1-N-phenylnaphthylamine (NPN), a validated marker for outer membrane permeability of GNRs (37), verified that sub-MIC concentrations of colistin or LL-37 increased outer membrane permeability of the MDR-*AB* strain (**Fig S 4.3**). Interestingly, AZM pretreatment of MDR-*AB* significantly enhanced binding of TAMRA-tagged LL-37 to the bacterial outer membrane, suggesting bidirectional synergy (**Fig S 4.4**).

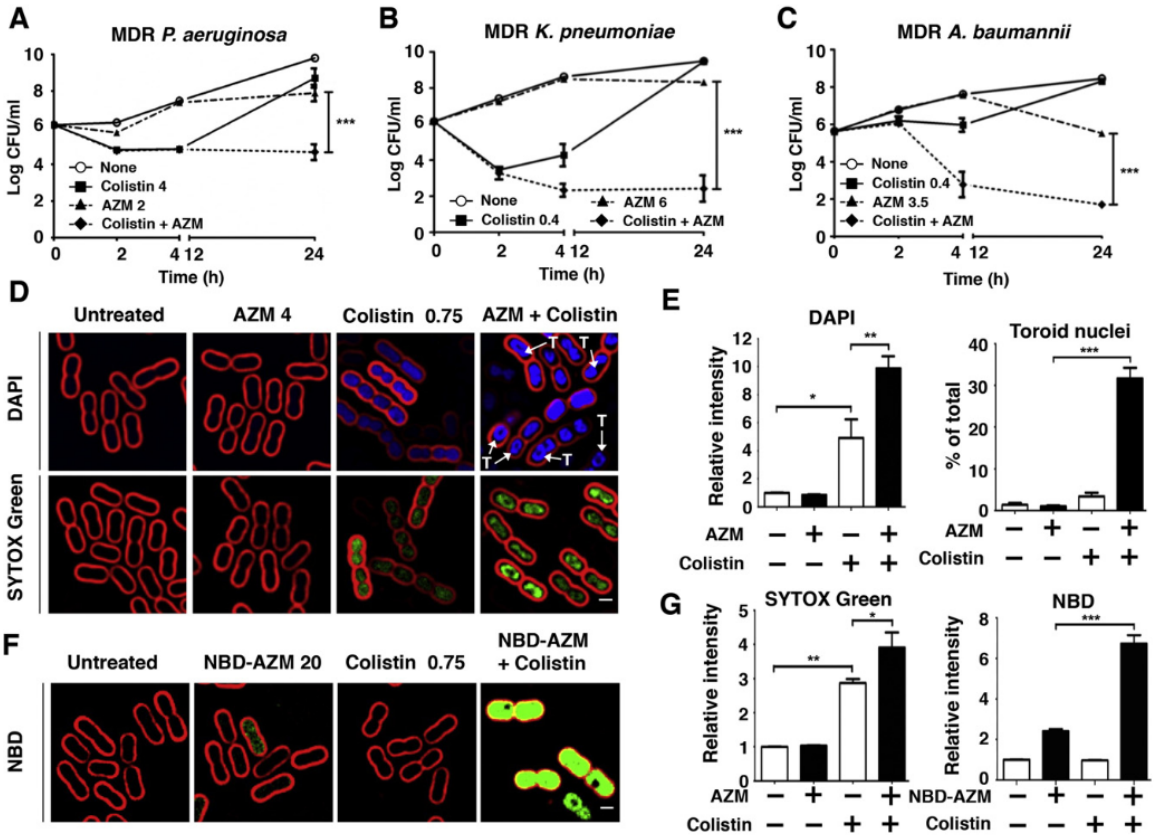


Figure 4.2 Colistin synergizes with AZM by increasing GNR outer membrane permeability. All assays conducted using the bacteriologic media Ca-MHB. (A-C) Time-kill curves demonstrate the effect of AZM, colistin, or both in combination against MDR *PA*, *KP* and *AB*. Data plotted are mean ± SEM and represent the average of triplicates from 3 independent experiments. ***P < 0.001; two-way ANOVA. (D and F) Growth phase MDR *AB* (5 x 10^7 CFU/ml) treated for 1 h with AZM (unlabeled or NBD-tagged), colistin, or a combination of both, then stained for fluorescence microscopy: FM4-64 (red cell membrane stain), DAPI (blue DNA stain), SYTOX Green (green DNA stain), and NBD-AZM (green NBD-tagged AZM), "T" = toroid shaped nucleoid. DAPI blue DNA stain present in "Untreated" and "AZM 4" panels. Since signal from all conditions was normalized to untreated controls and "AZM + Colistin" treated bacteria had 10-fold higher DAPI signal intensity, blue signal was reduced equally across all four displayed DAPI panels to prevent washout and enable visualization of the toroid structures in the cells with high DAPI signal intensity. (E and G) Bar graphs were generated from unbiased software analysis of multiple random microscopy fields with >500 cells counted per condition per experimental replicate. Data representative of 3 independent experiments and plotted as the mean ± SEM - additional microscopy details in methods section. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; one-way ANOVA.

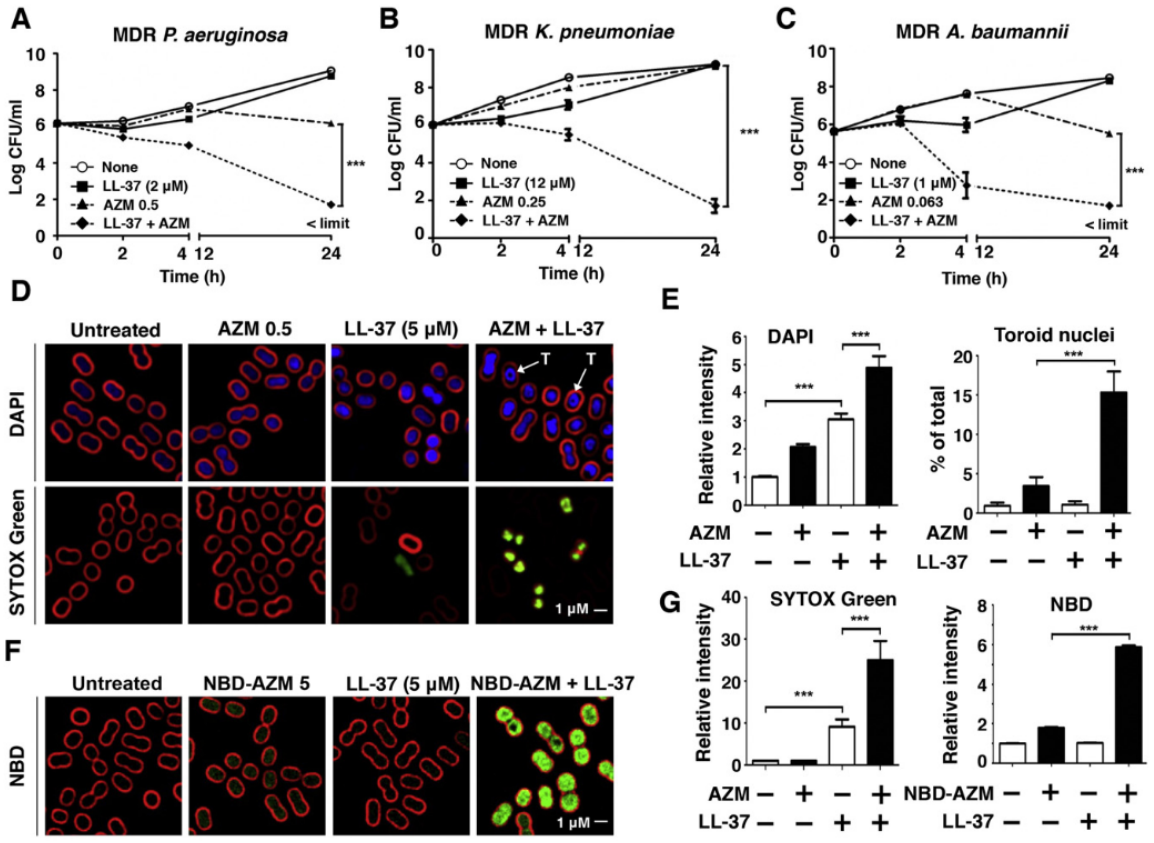


Figure 4.3 Cationic AMP LL-37 synergizes with AZM by increasing the outer membrane permeability of MDR-GNRs. All assays conducted using the eukaryotic media RPMI + 5% LB. (A-C) Time-kill curves demonstrating effects of AZM, LL-37, or both in combination against MDR *PA*, *KP*, and *AB*. Data plotted are mean ± SEM and represent the average of triplicates from 3 independent experiments. ***$P < 0.001$; two-way ANOVA. Limit of detection = 50 CFU/ml (D and F) Logarithmic growth phase MDR *AB* ($5x10^7$ CFU/ml) were treated for 2 h with AZM (unlabeled or NBD-tagged), LL-37, or a combination of both, then stained for fluorescence microscopy: FM4-64 (red cell membrane stain), DAPI (blue DNA stain), SYTOX Green (green DNA stain), and NBD-AZM (green NBD-tagged AZM), "T" denotes toroid shaped nucleoid. (E and G) Bar graphs generated from unbiased software analysis of multiple random microscopy fields with >500 cells counted per condition per experimental replicate. Data representative of 3 independent experiments and plotted as the mean ± SEM. - additional microscopy details in methods section. ***$P < 0.001$; one-way ANOVA.

3.5. AZM Monotherapy Promotes Clearance of MDR GNR in Murine Infection Models

Activity in mammalian culture media and synergy with host AMPs suggested AZM could be effective against MDR-GNR pathogens *in vivo*. AZM concentrates in soft tissues and phagocytes (38, 39), pharmacodynamic features providing high drug levels at infectious foci where endogenous cationic AMPs are deployed. We studied an intratracheal infection model of MDR-*AB* pneumonia with AZM as the sole therapeutic agent. A single subcutaneous dose of 50 or 100 mg/kg AZM was chosen to achieve serum concentrations mimicking those found with typical 500 or 1,000 mg AZM intravenous dosing given to human patients (22). A single AZM dose reduced by 2 $\log_{10}$ or 99% the amount of MDR *AB* recovered from lungs 24 h after challenge (**Fig 4.4A**). Examination of bronchial alveolar lavage fluid (BALF) revealed that AZM-treated animals had 50% fewer neutrophils (alveolar macrophages unchanged) (**Fig 4.4B**), significantly reduced pro-inflammatory cytokines IL-1β, IL-6, and MIP-2 (**Fig 4.4C**), and histological evidence of reduced neutrophil and bacterial infiltrations (**Fig 4.4D and E**). Increasing the inoculum of MDR-*AB* 10-fold produced significant mortality within 48 h. One dose of 100 mg/kg AZM after initial infection, followed by a second dose at 24 h, improved 5 d survival from 22% to 89% (**Fig 4.4F**). In a subcutaneous foreign body infection model, daily AZM reduced by >90% the quantity of MDR-*AB* recovered after 3 d (**Fig 4.4G**). Reductions of up to 10-fold in bacterial counts were also observed for AZM monotherapy in both MDR-*KP* and -*PA* lung infection models (**Fig 4.4H and I**). Low dose colistin therapy alone did not lead to a significant reduction in the amount of MDR *AB* recovered from lungs 24 h after challenge. However, when a low dose of AZM was combined with a low dose of colistin, a significant reduction in lung bacterial CFU was achieved (**Fig S 4.5**).

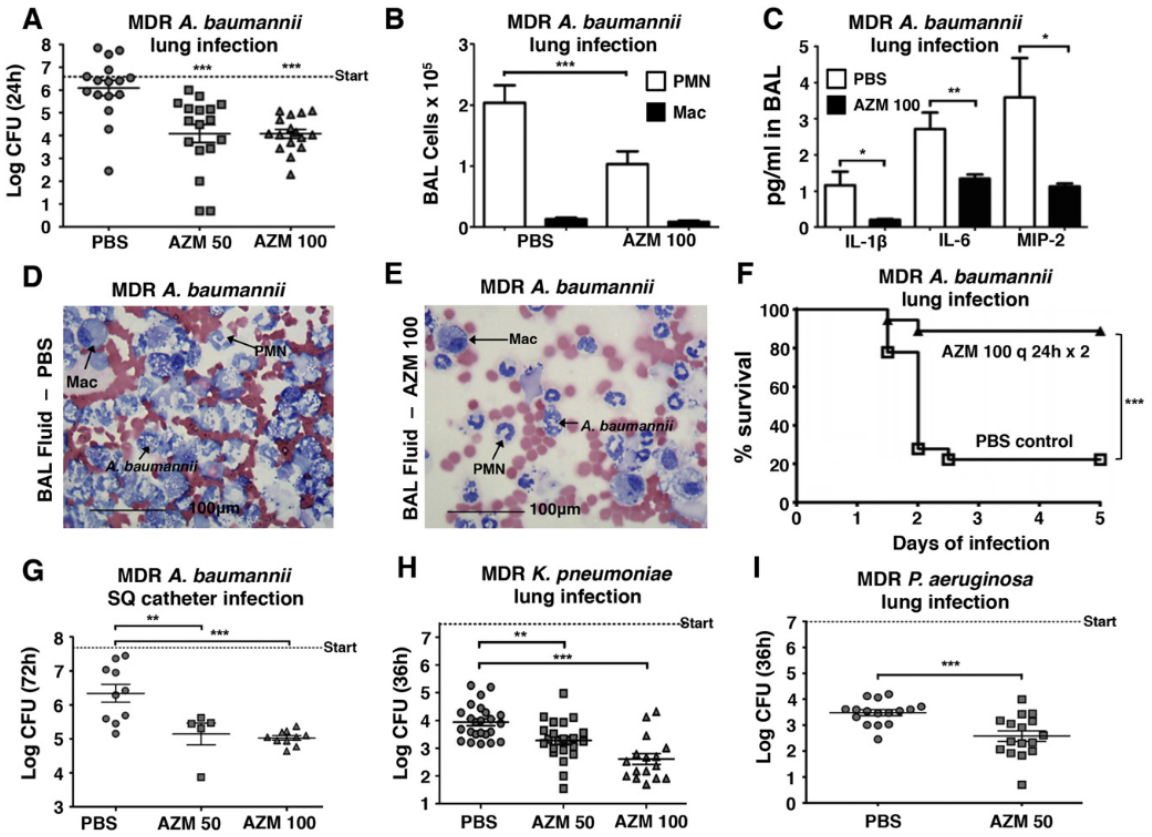


Figure 4.4 AZM activity as monotherapy against MDR-GNRs *in vivo*. (A) C57BL/6J mice were infected intratracheally (i.t.) with MDR *AB*. Lungs homogenized at 24 h; n = 16 for PBS control, 17 for AZM 50 mg/kg, and 16 for AZM 100 mg/kg. (B) Wright-Giemsa stained BALF from C57BL/6J mice infected i.t. with *AB*. Total neutrophils and macrophages enumerated by light microscopy + hemocytometer counts; n = 19 PBS control and 20 AZM 100 mg/kg-treated mice. PMN = polymorphonuclear leukocyte/neutrophil, Mac = macrophage. (C) ELISA detection of inflammatory cytokines in the BALF of mice from (b). (D and E) Light microscopy of Wright-Giemsa stained BALF of mice from (b); Mac = alveolar macrophage; PMN = neutrophil. (F) Survival of C57BL/6J mice infected i.t. with $6x10^7$ CFU of *AB*. Mice received two total doses of AZM versus PBS spaced 24 h apart. n = 18 PBS control and 18AZM 100 mg/kg-treated mice. (G) 1 cm catheter fragment coated with *AB* was implanted subcutaneously into CD-1 mice; results of 2 independent experiments. N = 10 PBS; 5 AZM 50 mg/kg-treated, 10 Az 100 mg/kg-treated mice (H) CD-1 mice infected i.t. with MDR *KP*. Lungs homogenized at 36 h. N = 24 PBS control, 23 AZM 50 mg/kg-treated, and 16 AZM 100 mg/kg-treated mice (i) CD-1 mice infected i.t. with MDR *PA*. Lungs homogenized at 36 h; results of 2 independent experiments. n = 16 PBS control and 16 AZM 50 mg/kg-treated mice. Data plotted as mean ± S.E.M. of 3 independent experiments unless otherwise stated. For *in vivo* mouse studies, AZM was dosed subcutaneously once every 24 h. $*P < 0.05$ $**P < 0.01$ $***P < 0.001$; one-way ANOVA *in vivo* studies, log-rank test for survival, two-tailed student's t test *in vitro* studies.

DISCUSSION

The continual emergence and rapid spread of MDR-GNRs in hospitals around the world has alarmed physicians, public health epidemiologists and government agencies, spurring urgent calls to action (3, 4). Due to historical precedent and the intrinsic appeal of a “gold standard”, a single bioassay, the MIC performed in bacteriological media, has come to dominate evaluation of antibiotic efficacy, from the earliest stages of the drug development process, to management of patients based on clinical isolate testing, to establishment of hospital formularies. However, even before the first diagnostic encounter with a physician, a patient’s infection is already being combatted by numerous endogenous antimicrobial components, including cationic AMPs of the innate immune system. We believe there is value in analyzing the action of pharmaceutical antibiotics in the richer context of these host defense factors.

Using traditional susceptibility testing methods, the familiar antibiotic AZM shows negligible activity against MDR-GNRs. A small number of prior studies examining multiple combinations of antibiotics in vitro have provided clues that AZM can exhibit activity against GNRs (40, 41) or synergize with a polymyxin type antibiotic (42), but without mechanistic or *in vivo* analysis or suggestions of clinical application. To this day, AZM remains excluded from the antibiotic testing panel reported to physicians when such bacteria are recovered from the blood, sputum or urine of hospitalized patients. Here we show that AZM has potent bactericidal activity against representative strains of the most fearsome MDR-GNRs when tested in eukaryotic cell media (+/- human serum) and *in vivo* murine models of infection. AZM entry and activity against MDR-GNRs is synergistically enhanced when the bacterial outer membrane is perturbed by cationic human AMP LL-37 or by the last-line antibiotic colistin. Because mutation of the *oprM* efflux pump system in *P. aeruginosa* has been associated with increased AZM sensitivity, and protein synthesis inhibition by AZM can reduce oprM gene expression (11), the enhanced entry of AZM in eukaryotic media and in synergy with colistin or LL-37 that we have demonstrated may initiate a positive feedback loop to increase effective intracellular levels of the antibiotic.

Of note, AZM is touted to have anti-inflammatory effects in lung tissues during infection (43), and several studies of short- or long-term AZM administration in patients with cystic fibrosis, a disease characterized by chronic recurrent pulmonary infection with *PA* and other MDR-GNR bacterial pathogens, have shown reductions in exacerbations and improvement in respiratory capacity (reviewed in (44). Our findings raise the possibility that these benefits could reflect, at least in part, a direct and otherwise unanticipated bactericidal activity of the drug. The macrolides, and azithromycin in particular, have a number of reported non-bactericidal properties that could further complement the *in vivo* efficacy we observed in our murine models of infection. For example, against various Gram-negative organisms, AZM has been shown to impair bacterial biofilms (45, 46), virulence factor production (45), motility (47), quorum sensing (21), and adherence to host epithelial cells (48, 49).

The major limitation of this study was the use of murine models of MDR GNR infection as opposed to human clinical cases. Laboratory mice are relatively resistant to infection by human clinical isolates of MDR GNRs requiring high inocula to create a disease phenotype. Furthermore, young mice with normal immunity were tested, whereas many human patients that develop MDR GNR infection have multiple co-morbidities including weakened immunity (e.g. from cancer chemotherapy) or dependence on a medical device (e.g. mechanical ventilator). Furture randomized clinical trials will be needed to validate whether or not AZM has therapeutic efficacy in older and sicker human patients suffering from MDR GNR infections.

In summary, our studies provide an experimental rationale to further explore AZM as adjunctive therapy in MDR-GNR infections. In particular, we have demonstrated for the first time that colistin or LL-37 permeabilization of Gram-negative outer membranes facilitates entry of the large AZM molecule. Potentially, additional antibiotics, currently disregarded for various clinical indications due to poor penetration into Gram-negative bacterial membranes in standard MIC testing, may likewise have unrecognized *in vivo* activities when host immune factors perturb bacterial membrane integrity. Most immediately, the AZM synergy with colistin we demonstrate

here may allow lower dose, colistin-sparing regimens that reduce adverse drug effects. Continued examination of pharmacodynamic interactions between administered antibiotics and endogenous AMPs of the innate immune system may reveal novel treatment strategies for challenging infections.

SUPPLEMENTAL MATERIALS AND METHODS

Serial Passage of MDR GNR isolates. Cultures of *Pseudomonas aeruginosa*, strain P4, *Klebsiella pneumoniae*, strain K1100, and *Acinetobacter baumannii*, strain AB5075 were grown at 1/2 and 1/4 their AZM MIC in 5 ml of RPMI + 5% LB. Cultures were placed in a 37^0C shaker, and 24 h later, the highest concentration of AZM in which dense bacterial growth was observed was recorded. 50 µl of this dense culture growth was then used to inoculate three new tubes containing 5 ml of fresh media and 2, 1, and 1/2 the original concentration of AZM respectively. Serial passage was performed twice and data represent one of two independent experiments.

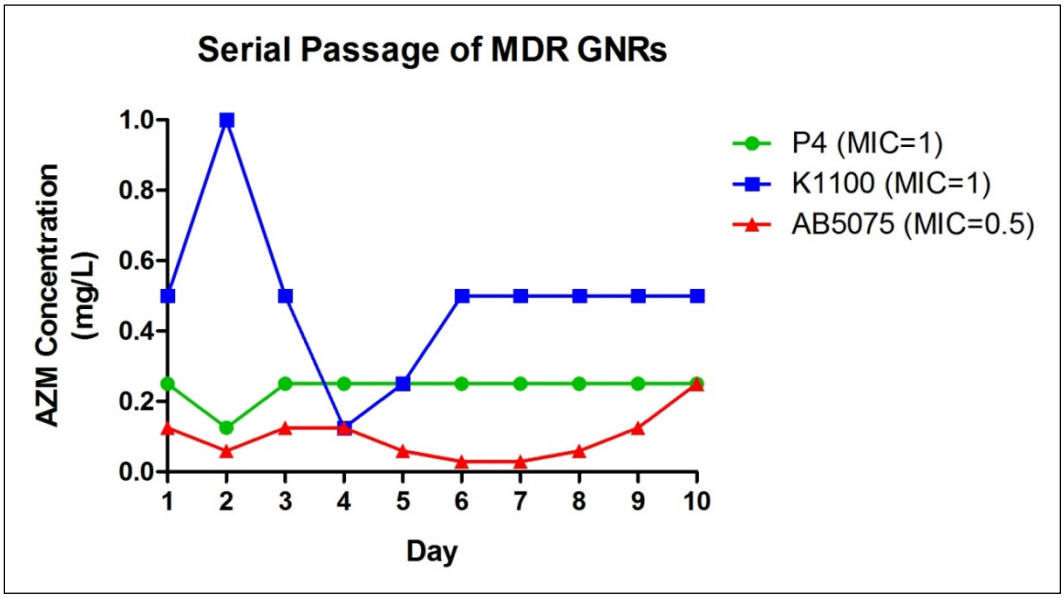
NPN bacterial outer membrane permeability assay. 1-N-phenylnaphthylamine (NPN) is a molecule that becomes fluorescent when it comes into contact with the inner membrane of Gram-negative bacteria, and this property was used to study MDR-*AB* membrane permeability (37). Overnight cultures of MDR-*AB* grown in LB at 37^oC with shaking were washed twice with PBS via centrifugation at 3220 x *g* at room temperature and re-suspended to an OD_{600} = 0.40 in 8 ml of Ca-MHB for colistin assays or 8 ml of 5% LB-RPMI, for LL-37 assays. LL-37, colistin, or media control was added to a final concentration of 1 μM and 0.5 mg/L respectively. The cultures were shaken at 37^oC for 1 h and then spun at 3000 x *g* at room temperature for 5 min and re-suspended in 2 ml of 10 mM Tris buffer pH 8.0. The concentrated 2 ml cultures were used to prepare 4 ml bacterial stocks at OD_{600} = 0.40 in 10 mM Tris. Assays were conducted in a final volume of 200 µl in triplicate in 96-well round bottom plates (Costar). Four conditions were tested. 1) 100 µl of bacterial stock + 50 µl of NPN (40 µM final) + 50 µl of 10 mM Tris. 2) 100 µl of

bacterial stock + 50 µl of NPN + 50 µl of EDTA (10 µM final). 3) 100 µl of bacterial stock + 50 µl of EDTA + 50 µl of 10 mM Tris. 4) 100 µl of 10 mM Tris + 50 *µ*l of NPN + 50 *µ*l of EDTA. As soon as all of the components were added and mixed, plates were immediately read in a fluorescent plate reader: excitation 250 nm / emission 420 nm. The NPN fluorescence signal from conditions 3) and 4), background, were subtracted from the signals measured from conditions 1) and 2). The NPN intensity from condition 1) bacteria + NPN was divided by the NPN signal measured from condition 2) bacteria + NPN + EDTA to obtain the percentage of permeability recorded in the presence of 10 mM EDTA that permeabilizes the outer membrane of Gram-negative bacteria.

***A. baumannii* foreign body infection model**. The *AB* foreign body infection studies were performed as previously described (24). Briefly, 14-gauge sterile non-pyrogenic intravenous catheters (Excel International) were cut into 1 cm segments. These segments were then cut in half, so that the interior lumen was opened. Catheter fragments were sterilized by shaking in 70% ethanol for 2 h and then rinsed twice in fresh LB. Catheter fragments were added to a 25 ml starting culture of AB5075 in LB and grown overnight at 37^{o}C with shaking. 8-week-old female CD1 mice (Charles River Labs) had the hair on their backs shaved and then removed with Nair (Naircare). The mice were anesthetized in an isoflurane chamber, their nude backs were sprayed with 70% ethanol, and a small 1 cm incision was made. Tweezers were gently inserted into the incision site and used to open up a small subcutaneous pouch. A catheter fragment was removed from the overnight culture of MDR-*AB* and inserted into the pouch. The wound site was closed with Tegaderm film (3M) and the mice were placed on a warm heating pad to recover. At 1, 24, and 48 h after infection, the mice received a subcutaneous dose of 50 mg/kg AZM, 100 mg/kg AZM, or 100 µl PBS control. At 72 h the mice were sacrificed with CO_2. The catheter fragment and all surrounding infected soft tissue was removed and placed in a 2 ml sterile micro tube (Sarstedt) containing 1 ml of PBS and 1 mm silica beads (Biospec) and the tissue was homogenized as detailed above for the lungs. Aliquots from each tube were serially diluted for

CFU enumeration on LB plates. Three catheter segments per experimental run were removed from the overnight *AB* culture and homogenized in 1 ml of PBS to determine the initial inoculum.

NBD-tagged AZM. 9a-NBD-AZM was synthesized as previously described (32) with slight modifications made in the final step of the synthesis. To a solution of 9a-(3-aminopropyl)-9-deoxo-9a-aza-9a-homoerythromycin A (100 mg, 0.126 mmol) in dry EtOH (2 ml) was added 4-chloro-7-nitrobenofurazan (25 mg, 0.126 mmol) and the resulting reaction mixture was stirred at room temperature for 5 h. Water (5 ml) was added to the reaction mixture and extracted with CH_2Cl_2 (3 x 5 ml). The organic layers were combined, dried over Na_2SO_4 and concentrated under reduced pressure. Flash chromatography (CH_2Cl_2 to 10:1 CH_2Cl_2/MeOH) afforded the desired compound as a yellow solid (66 mg, 55.1%). NBD-tagged AZM: ^{1}H NMR (CD_3OD, 500 MHz) δ 8.51 (d, *J* = 8.8 Hz, 1H), 6.42 (d, *J* = 9.0 Hz, 1H) –only the diagnostic benzofurazan shifts were noted (**Fig S 2.6A**); ^{13}C NMR (CD_3OD, 125 MHz) δ 177.7, 137.3, 102.1, 98.7, 95.6, 83.4, 79.0, 77.7, 77.1, 74.8, 74.5, 74.2, 73.0, 70.5, 67.2, 65.2, 64.5, 48.5, 44.9, 41.7, 40.9, 40.6, 38.8, 34.6, 34.3, 31.7, 31.4, 30.1, 29.4, 28.8, 28.2, 26.2, 24.8, 22.7, 22.3, 21.6, 21.1, 20.4, 20.2, 17.6, 16.4, 14.5, 13.1, 10.1, 8.8, 7.6; HRMS *m/z* calculated for $[C_{46}H_{79}N_6O_{15}]^+$: 955.5598, found 955.5597 (**Fig S 2.6B**).



Day	***Pseudomonas aeruginosa*, Strain P4 (MDR)** AZM (mg/L)	***Klebsiella pneumoniae*, Strain K1100 (MDR, KPC)** AZM (mg/L)	***Acinetobacter baumannii*, Strain AB5075 (MDR)** AZM (mg/L)
1	0.25	0.5	0.125
2	0.125	1	0.06
3	0.25	0.5	0.125
4	0.25	0.125	0.125
5	0.25	0.25	0.06
6	0.25	0.5	0.03
7	0.25	0.5	0.03
8	0.25	0.5	0.06
9	0.25	0.5	0.125
10	0.25	0.5	0.25

Abbreviations: AZM = Azithromycin; MDR = multidrug-resistant; KPC = *Klebsiella pneumoniae*, carbapenemase-producing; RPMI = Roswell Park Memorial Institute 1640, a basal mammalian tissue culture medium; LB = Luria broth.

Figure S 4.1 Serial passage for 10 days of MDR GNR isolates at sub-minimum inhibitory concentrations of azithromycin to test for resistance evolution. On Day 0, bacteria were grown at 1/2 and 1/4 of their AZM MIC in 5 ml of liquid culture - RPMI + 5% LB. Then 24 h after shaking at 37^0C, the highest concentration of AZM in which dense bacterial growth was observed was recorded. 50 µl of this dense culture growth was then used to inoculate 3 new tubes containing 5 ml of fresh media and 2, 1, and 1/2 the original concentration of AZM respectively.

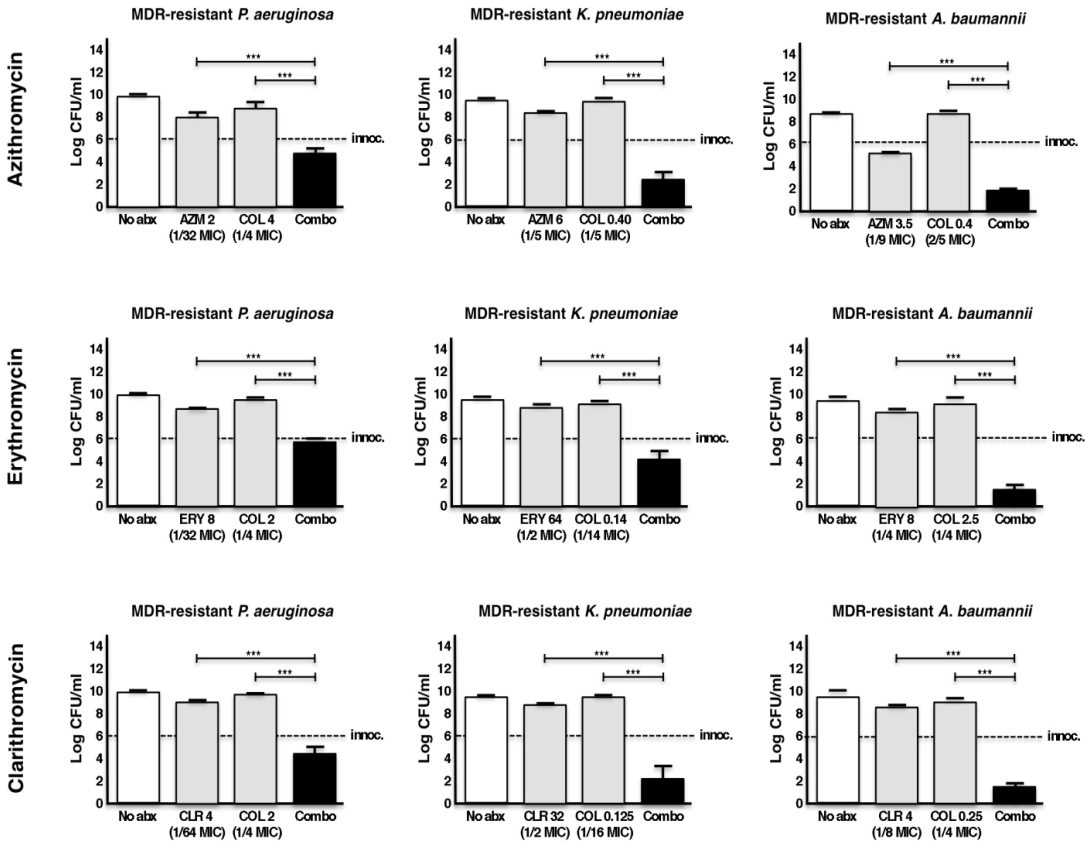


Figure S 4.2 Colistin synergizes with azithromycin, erythromycin, and clarithromycin to kill MDR GNR pathogens *in vitro*. All assays conducted using the standard bacteriologic media Ca-MHB. Time-kill assays demonstrate the effect of AZM, ERY, and CLR alone or in combination with colistin against MDR PA, KP and AB. Data plotted are mean ± SEM and represent the average of triplicates from two independent experiments. The starting bacterial innoculum ("innoc") is denoted by the dotted line. ***$P < 0.001$; one-way ANOVA.

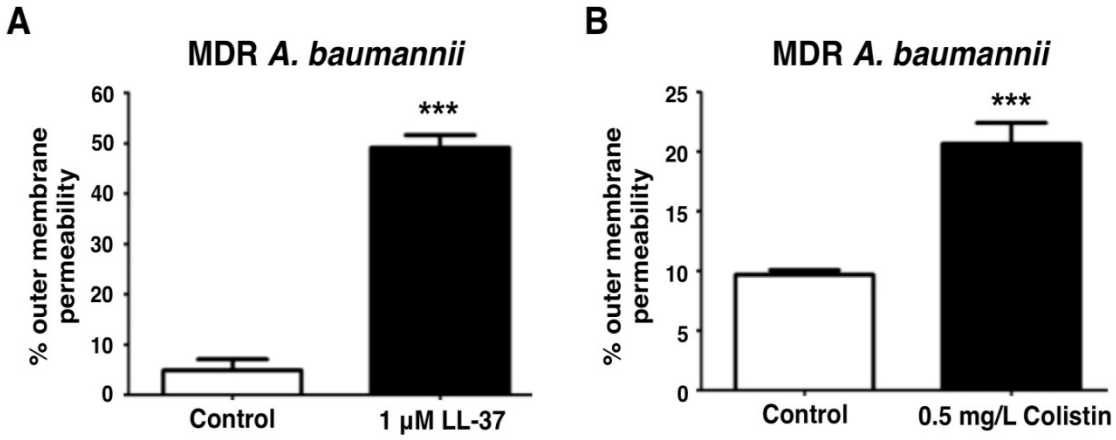


Figure S 4.3 Treating MDR *A. baumannii* with sub-MIC LL-37 or colistin significantly increases outer membrane permeability to 1-N-phenylnaphthylamine (NPN). Logarithmic growth phase MDR *AB* were treated for 1 h with 1 µM LL-37 in RPMI + 5% LB (**A**) or for 1 h with 0.5 mg/L colistin in Ca-MHB (**B**). Bacteria were then washed with 10 mM Tris buffer and treated with 40 µM NPN. Data are expressed as % of the maximal value recorded in the presence of 10 mM EDTA and represent the average of triplicates from 3 independent experiments. ***$P < 0.001$; two-tailed student's t-test.

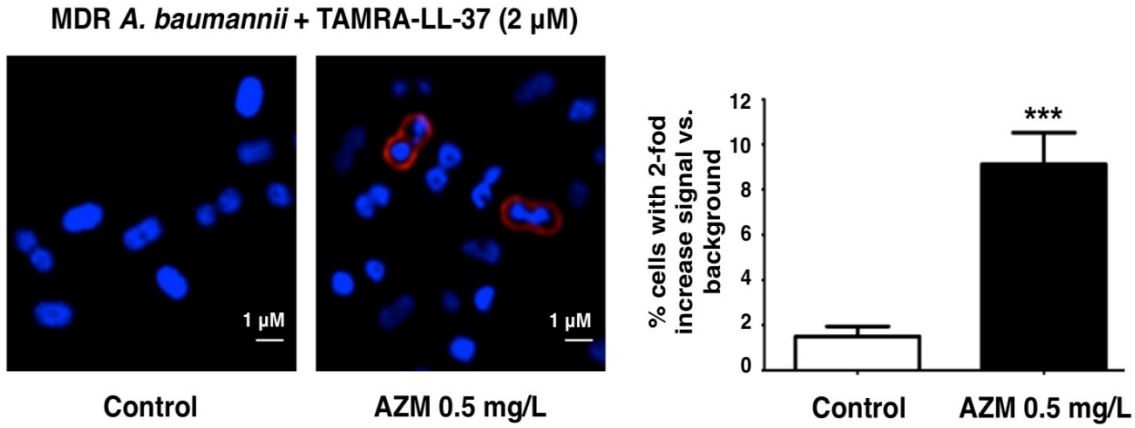


Figure S 4.4 Pre-treating MDR *A. baumannii* with AZM increases outer membrane binding of LL-37. Logarithmic growth phase MDR *AB* in RPMI + 5% LB were incubated for 2 h + 0.5 mg/L AZM. Bacteria were then treated for 30 min with TAMRA-tagged LL-37 (red), washed, and then stained with DAPI (blue DNA stain). The number of MDR *AB* with red membrane signals at least twice the background level were counted using unbiased software analysis of multiple random microscopy fields with >500 cells counted per experimental replicate. Data plotted as the mean ± SEM and represent the combination of 3 independent experiments. ***$P < 0.001$; two-tailed student's t-test.

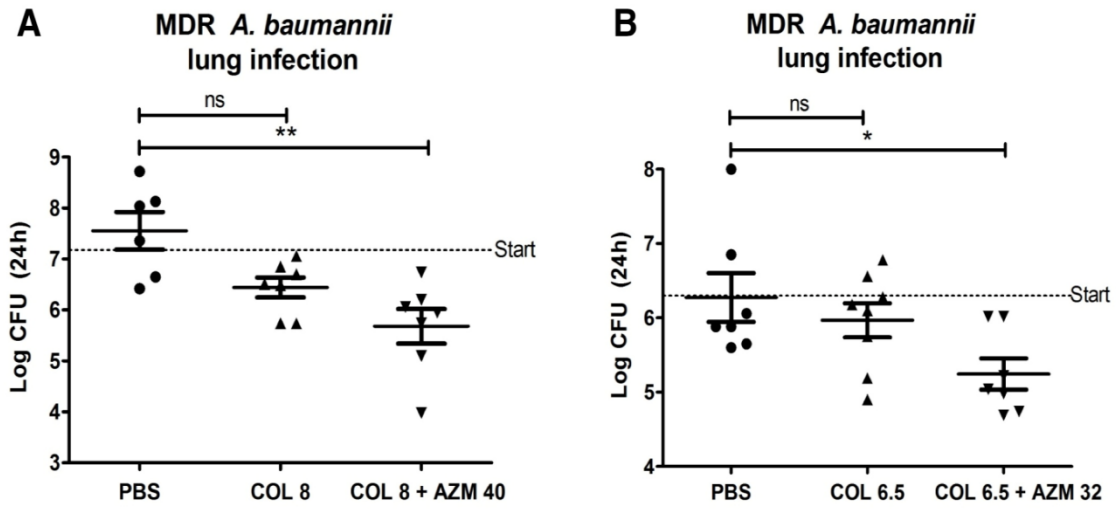


Figure S 4.5 Addition of low dose AZM to reduced dose colistin in treatment of MDR GNRs *in vivo*. (A) 8 wk old female C57BL/6J mice were infected intratracheally (i.t.) with MDR *A. baumannii* strain AB5075. Lungs were homogenized at 24 h; n = 6 for PBS control, 7 for colistin 8 mg/kg, and 7 for AZM 40 mg/kg ice. (B) 8 wk old female C57BL/6J mice were infected intratracheally (i.t.) with MDR *AB*. Lungs homogenized at 24 h; n = 7 for PBS control, 8 for colistin 6.5 mg/kg, and 7 for AZM 32 mg/kg ice. Data plotted as mean ± S.E.M. AZM was dosed once subcutaneously immediately after bacterial inoculation. Colistin was dosed once subcutaneously 1 h after the bacterial inoculation. Dashed line "start" denotes the initial inoculum. *$P < 0.05$ and **$P < 0.01$ based on one-way ANOVA analysis.

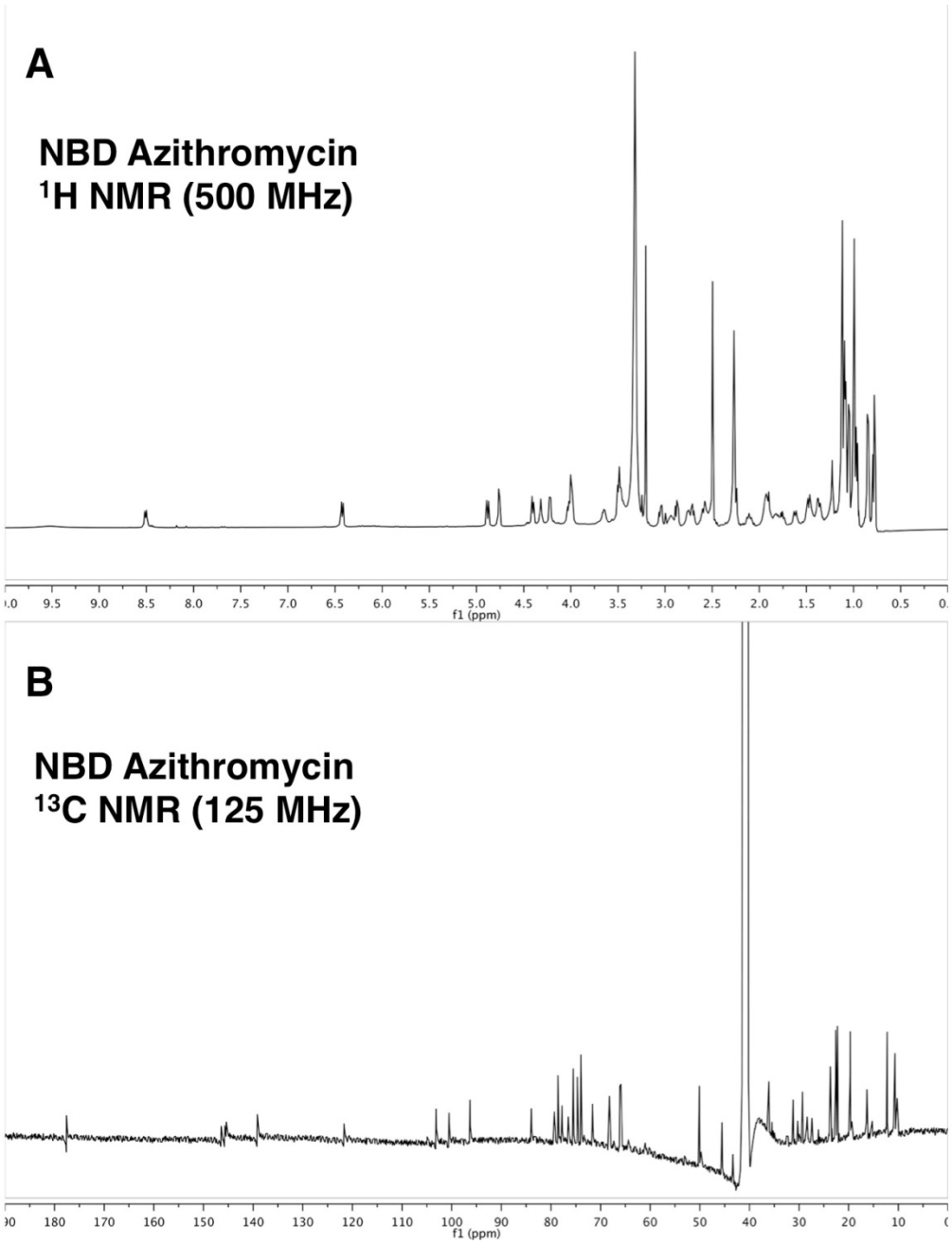


Figure S 4.6 Confirmation of structure and purity of synthesized NBD-tagged AZM. Shown are the spectra of (**A**) 1H NMR (500 MHz) and (**B**) 13C NMR (125 MHz) of NBD- tagged AZM in CD3OD.

Table S 4.1 Three multidrug (including carbapenem)-resistant Gram-negative bacterial strains examined in this study. Automatic minimum inhibitory concentration (MIC) testing results in cation-adjusted Mueller-Hinton broth by VITEK - 2 testing system.

ANTIBIOTIC	*P. aeruginosa*, P4 (MDR)		*K. pneumoniae*, K1100 (MDR, KPC)		*A. baumannii*, AB5075 (MDR)	
	MIC	Interpret	MIC	Interpret	MIC	Interpret
Ampicillin	≥ 32	R	≥ 32	R	≥ 32	R
Amoxicillin/Clav.	≥ 32	R	≥ 32	R	≥ 32	R
Ampicillin/Sul.	≥ 32	R	≥ 32	R	≥ 32	R
Ticarcillin			≥ 128	R	≥ 128	R
Ticarcillin/Clav.	≥ 128	R				
Pipericillin	≥ 128	R	≥ 128	R	≥ 128	R
Pipericillin/Taz.	≥ 128	R	≥ 128	R	≥ 128	R
Cefalotin	≥ 64	R	≥ 64	R	≥ 64	R
Cefazolin	≥ 64	R	≥ 64	R	≥ 64	R
Cefuroxime	≥ 64	R	≥ 64	R	≥ 64	R
Cefuroxime Axetil	≥ 64	R	≥ 64	R	≥ 64	R
Cefotetan	≥ 64	R	8	R	≥ 64	R
Cefoxitin	≥ 64	R	32	R	≥ 64	R
Cefpodoxime	≥ 8	R	≥ 8	R	≥ 8	R
Cefotaxime	≥ 64	R	8	R	≥ 64	R
Ceftazidime	≥ 64	R	≥ 64	R	≥ 64	R
Ceftizoxime	≥ 64	R	4	R	≥ 64	R
Ceftriaxone	≥ 64	R	≥ 64	R	≥ 64	R
Cefepime	≥ 64	R	4	R	≥ 64	R
Aztreonam	≥ 64	R	≥ 64	R	≥ 64	R
Doripenem	≥ 8	R	≥ 8	R	≥ 8	
Ertapenem			≥ 8	R		
Imipenem	≥ 16	R	8	R	≥ 16	R
Meropenem	≥ 16	R	≥ 16	R	≥ 16	R
Amikacin	32	I	≥ 64	R	≥ 64	R
Gentamicin	8	I	≥ 16	R	≥ 16	R
Tobramycin	≤ 1	S	≥ 16	R	8	I
Nalidixic Acid	≥ 32	R	≥ 32	R	≥ 32	R
Ciprofloxacin	≥ 4	R	≥ 4	R	≥ 4	R
Levofloxacin	≥ 8	R	≥ 8	R	4	I
Moxifloxacin	≥ 8	R	≥ 8	R	≥ 8	R
Norfloxacin	8	I	≥ 16	R	≥ 16	R
Tetracycline	≥ 16	R	4	S	≤ 1	S
Tigecycline	≥ 8	R	4	I	≤ 0.5	S
Nitrofurantoin	≥ 512	R	128	R	≥ 512	R
TMP/SFX	≥ 320	R	40	S	≥ 320	R

Abbreviations: MDR = Multidrug-resistant; KPC = *Klebsiella pneumonia,* carbapenemase-producing;
R = Resistant; I = Intermediate; S = Sensitive; TMP/SFX = Trimethoprim/Sulfamethoxazole

Table S 4.2 Minimum inhibitory concentration (MIC) of Gram-negative bacteria strains used in this study to azithromycin, ciprofloxacin, erythromycin, clarithromycin, colistin, and human cathelicidin LL-37. Comparative testing performed in standard bacteriologic media or mammalian tissue culture medium.

	Bacterial Species/Strain	*A. baumannii*, Strain AB5075 (MDR)	*A. baumannii*, Strain AB19606	*K. pneumoniae*, Strain K1100 (MDR, KPC)	*K. pneumoniae*, Strain K700603	*P. aeruginosa*, Strain P4 (MDR)	*P. aeruginosa*, Strain PA01
Azithromycin MIC (mg/L)	Ca-MHB	32	64	32	64	> 64	> 64
	RPMI + 5% LB	0.5	0.25	1	2	1	2
Ciprofloxacin MIC (mg/L)	Ca-MHB	> 25	2	> 25	< 0.5	12	0.8
	RPMI + 5% LB	> 25	3	> 25	< 0.5	12	0.8
Erythromycin MIC (mg/L)	Ca-MHB	32		128		>256	
	RPMI + 5% LB	1		4		8	
Clarithromycin MIC (mg/L)	Ca-MHB	32		64		>256	
	RPMI + 5% LB	0.5		4		8	
Colistin MIC (mg/L)	Ca-MHB	1		2		8	
	RPMI + 5% LB	2		2		4	
LL-37 MIC (μM)	RPMI + 5% LB	4		32		64	

Abbreviations: Ca-MHB = Cation-adjusted Mueller-Hinton broth; RPMI = Roswell Park Memorial Institute 1640, a basal mammalian tissue culture medium; LB = Luria broth; MDR = multidrug-resistant; KPC = Klebsiella pneumonia, carbapenemase-producing.

Table S 4.3 Minimum inhibitory concentration (MIC) of azithromycin vs. a panel of contemporary clinical isolates of multidrug-resistant Gram-negative bacteria. Comparative testing performed in standard bacteriologic testing media or mammalian tissue culture medium.

Bacterial Species/Culture Site	**Azithromycin** MIC (mg/L)	
	Ca-MHB	RPMI + 5% LB
Pseudomonas aeruginosa, WP2 (MDR) - Sputum	32	1
Pseudomonas aeruginosa, WP3 (MDR) - Wound	256	0.5
Pseudomonas aeruginosa, WP5 (MDR) - Sputum	32	<0.06
Pseudomonas aeruginosa, UCSD P1 (MDR) - Urine	128	2
Klebsiella pneumoniae, WK7 (MDR) - Urine	256	32
Klebsiella pneumoniae, WK8 (MDR) - Urine	32	1
Klebsiella pneumoniae, WK9 (MDR) - Urine	32	0.5
Klebsiella pneumoniae, WK10 (MDR) - Sputum	>256	32
Acinetobacter baumannii, WA2 (MDR) - Wound	32	0.125
Acinetobacter baumannii, WA4 (MDR) - Peri-anal	64	0.125
Acinetobacter baumannii, WA5 (MDR) - Sputum	64	0.125

Abbreviations: Ca-MHB = Cation-adjusted Mueller-Hinton broth; RPMI = Roswell Park Memorial Institute 1640, a basal mammalian tissue culture medium; LB = Luria broth; MDR = multidrug-resistant.

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CHAPTER 5

CONCLUSIONS

Multidrug-resistant Gram-negative bacteria pose a serious public health threat both at home and abroad. Their possession of a highly impermeable outer membrane, coupled with a large number of drug efflux pumps and antibiotic neutralizing enzymes, allow many Gram-negative bacteria to be inherently resistant to many of the pharmaceuticals currently used to treat such infections. A report commissioned by the UK government on antimicrobial resistance has estimated a sharp rise in deaths from current estimates of 700,000 deaths annually to 10 million deaths annually by 2050 if we do nothing to stem the rise of antimicrobial resistance (1). Economically, this would lead to a decrease in gross domestic product by up to 3.5% and economic losses in the $100 trillions globally. It is thus imperative that we begin to develop novel strategies to combat antibiotic resistance in Gram-negative bacteria, a subject that has been neglected in recent decades, in order to prevent the onset of a “post-antibiotic era”.

This dissertation project offers evidence that the *mla* pathway governing membrane lipid asymmetry may be a viable target to sensitize Gram-negative bacteria to outer membrane damage caused by components of the host innate immune system. Earlier, a number of research groups have documented the need for an intact *mla* pathway for Gram-negative bacteria to retain full virulence; the pathway is required for intracellular spread of *Shigella flexneri* (2, 3), resistance to certain antibiotics, ethanol, and surfactants in *Escherichia coli* (4-8), lung infection and serum resistance in *Haemophilus influenzae* (9, 10), phenol tolerance in *Pseudomonas putida* (11), colistin resistance in *Acinetobacter baumannii* (12-14), and maximal resistance to antibiotics and acidified nitrite in *Pseudomonas aeruginosa* (15-17). Our evidence advances the concept that pharmacological inhibition of the *mla* pathway could represent a novel strategy in treating Gram-negative infections. This final chapter of the dissertation will summarize the key discoveries of this work and suggest future studies to evolve this platform into a viable therapeutic agent.

SUMMARY OF RESULTS

At the beginning of this dissertation project in 2012, only few studies on the host-pathogen interactions of *A. baumannii* had been published. Many of these studies focused on the diverse ways in which *A. baumannii* resisted antibiotics (18) and a few virulence factors that had been identified, such as *ompA* (19, 20), biofilm-associated protein (21, 22), acinetobactin (23), polysaccharide capsule (24), and lipopolysaccharide (25). Limited research since then has been focused on strategies to sensitize Gram-negative bacteria to host innate immune responses. In one avenue of investigation, small molecule inhibitors have been identified that block *Pseudomonas aeruginosa* production of pyocyanin, which could theoretically sensitize bacteria to reactive oxygen species, but the majority of papers only carry out the analysis as far as quantifying reduced production of the putative virulence factor (26, 27).

We initiated our investigation by conducting a screen for *A. baumannii* genes that were important in resisting the bactericidal effects of the human cathelicidin antimicrobial peptide, LL-37. Of the 3900 random transposon mutants we tested, 8 mutants exhibited at least a 2-fold increased LL-37 sensitivity. The most promising of these mutants had a transposon insertion in the putative lipoprotein encoding a gene with homology to *vacJ* in other Gram-negatives, which is fully discussed in Chapter 2. Malinverni and Silhavy showed that VacJ and products of the Mla operon were a part of a single pathway that is involved in maintaining outer membrane lipid asymmetry (5). Transposon insertions in *mlaC*, *mlaE*, and *mlaE* demonstrated similar sensitivity to *vacJ* mutant in an LL-37 MIC assay. *vacJ* mutant also demonstrated increased sensitivity to the detergent SDS and increased permeability to the small fluorescent dye NPN upon damage with colistin. When challenged with components of the innate immune system such as whole blood, serum complement, and neutrophils, *A. baumannii vacJ* mutant had reduced ability to survive when compared to wild-type parent strain. In a mouse lung infection model, *A. baumannii vacJ* mutant infection resulted in reduced damage to alveolar architecture, bacterial burden,

bacteremia, and mortality. These results showed the importance of VacJ and the Mla pathway in order for *A. baumannii* to achieve full virulence.

Since the Mla pathway is highly conserved in Gram-negative bacteria, we sought to determine its role in infection in another Gram-negative opportunistic pathogen that is a major antibiotic-resistance threat. A similar 2-fold increase in sensitivity to LL-37 across *P. aeruginosa vacJ* and *mla* transposon mutants was observed in an MIC assay. A *P. aeruginosa vacJ* also demonstrated increased sensitivity to SDS in the presence of the cation chelator EDTA. The *P. aeruginosa vacJ* mutant has increased outer membrane permeability upon damage with LL-37 when measured with the NPN assay. In the presence of whole blood and human complement, the *P. aeruginosa vacJ* mutant was also unable to survive compared to wild-type parent strain. Mice infected with the *P. aeruginosa vacJ* mutant also demonstrated reduced bacterial load and bacteremia as well as increase survival when compared to a wild-type infection. These results helped to establish the broader importance of outer membrane stability to Gram-negative pathogens during the course of infection.

In Chapter 4, we examined the phenomenon in which a common macrolide antibiotic, azithromycin, becomes highly bactericidal against Gram-negative pathogens when tested using tissue culture media as compared to standard bacteriological testing media. Increased bactericidal action of azithromycin was due to the disruption of the highly impermeable outer membrane; in the presence of colistin or LL-37, azithromycin was able enter the cell and exhibit multi-log synergistic killing at very low concentrations. These results were translated into animal models of infection with highly drug-resistant Gram-negative pathogen infections in which azithromycin monotherapy was incredibly effective. This discovery highlights an immediate application of a safe, approved antibiotic for treatment of patients suffering multidrug-resistant Gram-negative bacterial infections, otherwise unrecognized by standard antimicrobial testing that ignores the innate immune system. This published chapter (28) helps solidify the theme in which Gram-negative bacterial pathogens require an intact outer membrane in order to fully resist innate

immune defensive actions and antibiotics that were previously shown to be ineffective under standard testing conditions.

FUTURE DIRECTIONS

This thesis dissertation has shown that inactivating the *mla* pathway may be a viable pharmacological target that can sensitize Gram-negative pathogens to killing by components of the innate immune system. Phenotypic effects were conserved between two different Gram-negative pathogens (*A. baumannii* and *P. aeruginosa*), and distillation of the current literature hints that a broad relevance may extend across all Gram-negatives. For example, antibodies raised against *Pasteurella multocida* recombinant VacJ are shown to be protective in a mouse model of infection (29) demonstrating that the *mla* system accessible to therapeutic targeting.

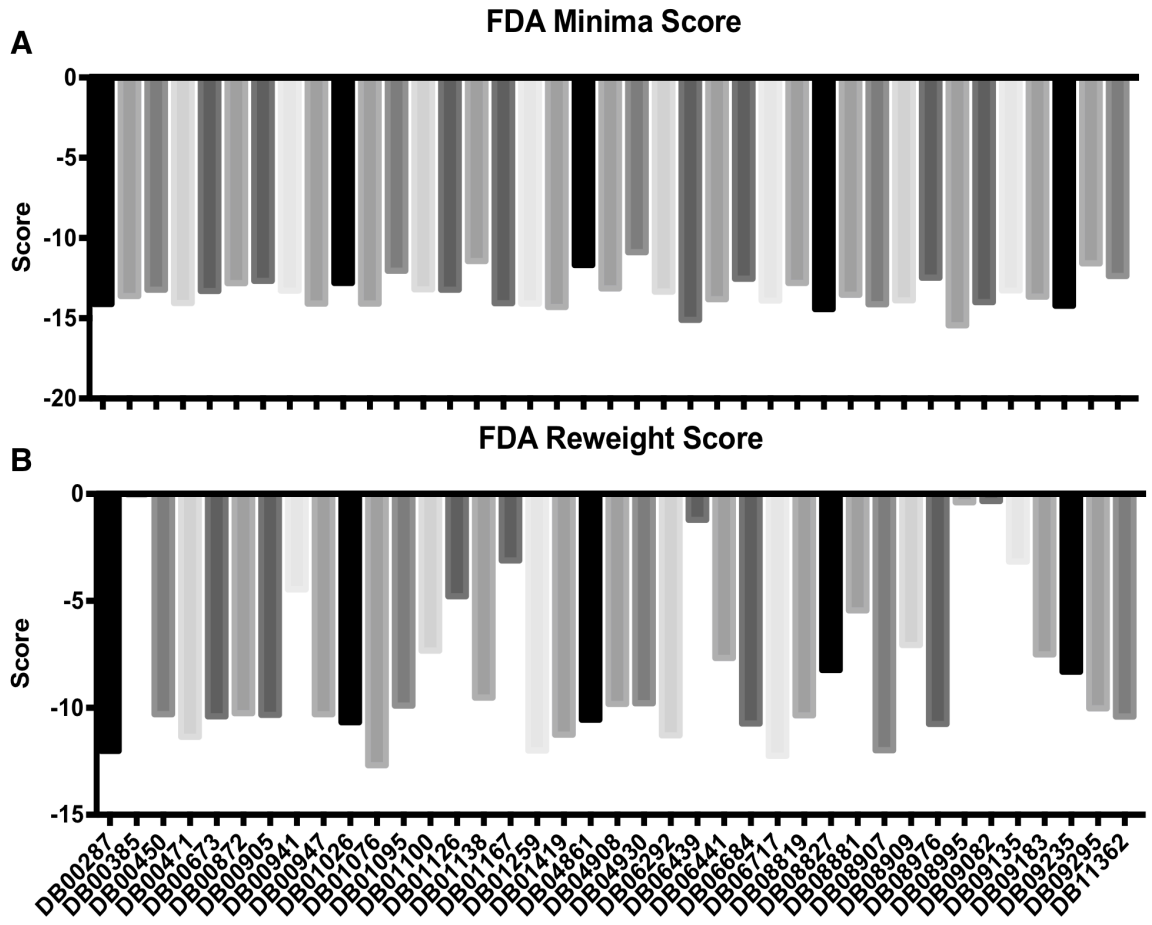


Figure 5.1 *In silico* screen of FDA compounds. (**A**) Best 39 scores from 1826 screened compounds in library. (**B**) Averaged scores of 39 compounds tested against 30 confirmations of MlaC.

In a collaboration with the J. Andrew McCammon lab in the UCSD Division of Chemistry & Biochemistry, we have begun studying the molecular dynamics of a published crystal structure of *Ralstonia solanacearum* MlaC (PDB 2QGU) interacting with phospholipids, to initiate effort toward discovery of pharmacological inhibitors of its action the could disrupt the *mla* pathway (30). We found by examining the protein sequence of *R. solanacearum* and *A. baumannii* an overall 29% homology, with many of the amino acids in the phospholipid-binding pocket conserved or possessing similar properties. Employing a virtual screen of FDA-approved compounds, we performed an *in silico* docking assay to identify small molecules that would fit into

the phospholipid binding pocket of MlaC in homology models of the corresponding *A. baumannii* and *P. aeruginosa* MlaC using the online program I-TASSER (31). From a virtual screen of 1826 FDA approved compounds, 39 compounds were selected for further study (**Fig 5.1A**). These 39 compounds were then run through 30 different MlaC confirmations and reweight scores were calculated (**Fig 5.1B**). Lowest scores obtained from these results achieved the highest priorities for testing in combination with LL-37 for sensitization of *A. baumannii* and *P. aeruginosa* to killing by the host defense peptide. Compound identities can be found in **Table 5.1**. An interesting observation can be seen with 2 separate hits, DB06717 (fosaprepitant) which is a prodrug of DB00673, aprepitant. Loss of a phosphonate led to an increase in score from -12.2 to -10.4, which translates into an increased energy requirement to fit into the phospholipid-binding pocket. Due to metabolic processing of fosaprepitant into aprepitant, priority for testing the compound would be given to other compounds.

Future studies will seek to characterize the ability of FDA compound hits to sensitize Gram-negative pathogens to LL-37, serum, and whole blood. Testing will be performed on *P. aeruginosa* and *A. baumannii,* whose predicted enzyme structures were used in the virtual screen, then extended to studies on other medically important and antibiotic-resistant Gram-negative pathogens. Top hits can then be prepared for testing *in vivo* using our established murine lung infection model for their ability to reduce bacterial load in the lung, prevent bacteremia, and increase mouse survival. In addition, promising compound hits can be tested in combination with last line of defense antibiotics in multidrug resistant Gram-negative pathogens to see if they have synergistic effects.

Pharmacological targeting the outer membrane lipoprotein VacJ could also be promising. Antiserum has already been generated by vaccination with recombinant *Pasteurella multocida* VacJ that affords passive protection in a mouse infection model (29). Mechanistically, this antiserum could be tested to see if it in fact sensitizes *P. multocida* to antimicrobial peptides and other membrane active compounds, as predicted by our research. This approach also be a way

of selectively targeting Gram-negative pathogens therapeutic antibodies for innate immune sensitization and synergy with colistin and related antibiotics.

Table 5.1 FDA compound leads

Ascension #	Name	Ascension #	Name	Ascension #	Name
DB00287	Travoprost	**DB01126**	Dutasteride	**DB08819**	Tafluprost
DB00385	Valrubicin	**DB01138**	Sulfinpyrazone	**DB08827**	Lomitapide
DB00450	Droperidol	**DB01167**	Itraconazole	**DB08881**	Vemurafenib
DB00471	Montelukast	**DB01259**	Lapatinib	**DB08907**	Canagliflozin
DB00673	Aprepitant	**DB01419**	Antrafenine	**DB08909**	Glycerol phenylbutyrate
DB00872	Conivaptan	**DB04861**	Nebivolol	**DB08976**	Floctafenine
DB00905	Bimatoprost	**DB04908**	Flibanserin	**DB08995**	Diosmin
DB00941	Hexafluronium	**DB04930**	Permethrin	**DB09082**	Vilanterol
DB00947	Fulvestrant	**DB06292**	Dapagliflozin	**DB09135**	Ioxilan
DB01026	Ketoconazole	**DB06439**	Tyloxapol	**DB09183**	Dasabuvir
DB01076	Atorvastatin	**DB06441**	Cangrelor	**DB09235**	Efonidipine
DB01095	Fluvastatin	**DB06684**	Vilazodone	**DB09295**	Talniflumate
DB01100	Pimozide	**DB06717**	Fosaprepitant	**DB11362**	Selexipag

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APPENDIX A

LL-37 SENSITIVITY SCREEN OF AB5075 RANDOM TRANSPOSON MUTANTS

The following were the protocol and results from the LL-37 selection screen on the multidrug-resistant *Acinetobacter baumannii* AB5075 random transposon mutant library.

LL-37 Sensitivity Selection Protocol

Day -1

1. Grow transposon mutant library overnights in 200 μL of Luria broth (LB) in 96-well round bottom plates (Costar) by inoculating with 1 μL of frozen overnight cultures.

Day 0

1. Grow transposon mutant library in LB for ~2 h (translates to mid-log phase) from overnight cultures by inoculating 1 μL into 200 μL of media.
2. Create enough media to fill 96-well round bottom plates with 199 μL of 2 μM LL-37 in RPMI + 5% LB.
3. Once LL-37 containing media is in the plates, add 1 μL of overnight transposon mutants, making sure to note locations of wells.
4. Place at 37 ºC overnight.

Day 1

1. Add 20 μL of resazurin (15.65 μg/mL) to each well and place back at 37 ºC.
2. Note which wells were unable to convert resazurin dye pink, translating to inability to either survive in 2 μM LL-37 or inability to grow in RPMI + 5% LB. Repeat until all plates are finished.

To sequence mutants, use overnight culture of *A. baumannii* transposon mutant. Perform two-stage semi-degenerative PCR using Kappa Hot Start High Fidelity DNA Polymerase (**Table A1**). Sequence with hyg-107 primer. Results of hits are found in **Table A2**. Primers used were:

primer hyg-174	GAA GCA TTT ATC AGG GTT ATT GTC TCA
CEKG 2A	GGC CAC GCG TCG ACT AGT ACN NNN NNN NNN AGA G
CEKG 2G	GGC CAC GCG TCG ACT AGT ACN NNN NNN NNN CTA G
CEKG 2H	GGC CAC GCG TCG ACT AGT ACN NNN NNN NNN TAA GT
hyg-154	TGT CTC ATG AGC GGA TAC ATA TTT GA
CEKG 4	CCG GTG CGC AGC TGA TCA TG
hyg-107	AAT AGG GGT TCC GCG CAC ATT

Table A1 PCR Protocol for obtaining products to sequence.

PCR Round 1			
Step	**Temperature**	**Time**	**Notes**
1	94 degrees	12'	Colony Denaturing
2	94 degrees	30"	Denaturing
3	42 degrees	30"	Annealing, decreased temp 1 degree each c
4	72 degrees	3'	Extension
5	go to step 2		6 cycles, then to step 6
6	94 degrees	30"	Denaturing
7	64 degrees	30"	Annealing
8	72 degrees	3'	Extension
9	go to step 6		25 cycles then step 10
10	72 degrees	7'	Final extension
11	4 degrees	hold	

PCR Round 2			
Step	**Temperature**	**Time**	**Notes**
1	94 degrees	5'	Initial Denaturing
2	94 degrees	30"	Denaturing
3	64 degrees	30"	Annealing
4	72 degrees	3'	Extension
5	go to step 2		30 cycles then step 6
6	72 degrees	7'	Final extension
7	4 degrees	hold	

Table A2 LL-37 Sensitivity Hits

AB5075 Tn Mutant	Seq	Gene	Function
1C9	GCATCAAATGACTATATCGAGGTGAATGATGCTGCTACGGGTGAAACTTTGGCATGGGTAAAAA	ABUW_0204	Succinate semialdehyde dehydrogenase
1D12	TATAGACAGCAATCATTAATGCGACAATCAAAATCACTGGACGGATGACTTTTTTCAACTCCAATTGAT	ABUW_3560	HlyD family secretion protein
2C12	Need to Resequence		
3D12	GAAGTAACATCTTCCGAACTAGCGGC	ABUW_3259	VacJ family lipoprotein
3H2	Need to Resequence		
27A11	Need to Resequence		
27A12	GATTTAAACATGCTCGAGCCTTACATTTACCTTTT	ABUW_0473	Hypotetical protein