2023

Molecular mechanisms underlying meropenem tolerance in Acinetobacter baumannii

Nowrosh Islam

Molecular mechanisms underlying meropenem tolerance in *Acinetobacter baumannii*

by
Nowrosh Islam

DISSERTATION
Submitted in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy

The University of Texas at Arlington
Arlington, Texas
December 2023

Supervising Committee:
Dr. Joseph M. Boll, Supervising Professor and Committee Chair
Dr. Cara C. Boutte, Committee Member
Dr. Todd A. Castoe, Committee Member
Dr. Piya Ghose, Committee Member
Dr. Matthew Walsh, Committee Member

COPYRIGHT

Dedication

To my parents Nazrul Islam and Nilufar yeasmin for their unwavering love, support and sacrifices throughout this journey, my husband Marnim Galib for his inspiration, patience and my daughter Umaiza Galib for being the biggest strength in my life.

ACKNOWLEDGEMENTS

At first, I would like to thanks to my mentor, Dr. Joseph Boll for his invaluable guidance, mentorship, and support throughout my research journey. Your insightful comments, constructive criticism, and unending encouragement has enhanced my critical thinking ability and shaped me as a researcher. Thanks to the members of my graduate committee, Dr. Cara C Boutte, Dr. Piya Ghosh, Dr. Todd Castoe and Dr. Matthew Walsh for their expert guidance, thoughtful feedback, and valuable suggestions. A special thanks to Dr. Cara C Boutte for her microscope training and for allowing me to work in her laboratory as a member in my last semester. I am thankful to all the current and former members of Boll Laboratory. A huge thanks to Dr. Misha Kazi for all the great brainstorming sessions and valuable suggestions, Dr. Katie Kang for all your help, Dr. Feroz Ahmed for being like a big brother and for helping me troubleshoot my experiments. Thanks to Sinjini Nandy for your cooperation and being like a little sister, Himani Meena for helping me making transposon library when I could not tolerate the filthy odor, Deborah Omoregie for being a super fun lab mate and Arshya Tehrani for tolerating my jokes and for numerous rides from campus to Mellow Mushrooms. I heartily thanks to my friends from Dr. Boutte Lab, Ghose lab and Mark lab for their generosity with equipment. Special thanks to Augusto Cesar Hunt Serracin for helping me with recombineering, Dr. Farah Shamma for being a big sister and always helping with my research, and Neda Habibi for her tips and tricks about western blot and all the fun-filled discussions. Lastly, I would like to say thanks to my friend outside biology department without whom my foreign life would be miserable. I am grateful to my friends Shamsad Nuri Mouri, Tasmia Eva, Imran Kabir, Reehan and Amayrah for making my PhD journey filled with joy and laughter when I was in stress. Finally, I am deeply grateful to my parents for being there for me in every way possible throughout my studies with their dedication and inspiration. Thanks to my sister, Nazia Islam and my two nieces Juhaina and Namira for their motivation and making me laugh with their silly jokes, to my in-laws for their great supports and encouragement and to my husband Marnim Galib whose generosity, patience and support enabled me to pursue my PhD. To my daughter Umaiza Galib, you are the biggest strength and support in my life. Thanks for being a good girl when I was busy with experiments and study. Without all of you, this journey would not be possible.

TABLE OF CONTENTS

ABSTRACT

MOLECULAR MECHANISMS UNDERLYING MEROPENEM TOLERANCE IN *ACINETOBACTER BAUMANNII*

Nowrosh Islam,
The University of Texas at Arlington, 2023

Supervising Professor: Joseph Boll

The emergence and proliferation of Gram-negative bacterial infections has become a serious public health concern due to their rapid rise of resistant to all the clinically available antibiotics. The increasing incidence of bacterial infections has prioritized the invention of new therapeutics to prevent the antibiotic treatment failure. Typically, Gram-negative bacteria use several defensive strategies such as modification of the cell envelope to escape the lethal effects of bactericidal antibiotics. Carbapenem beta-lactam considered last resort antibiotic to treat Gram-negative bacteria infections, while they are considered first line prescription against nosocomial pathogen *Acinetobacter baumannii* (denoted as *Ab*). Beside resistance, the susceptible populations of *Ab* show high tolerance to carbapenem antibiotic meropenem, which is an understudied potential contributor of treatment failure. Like Enterobacteriaceae, beta-lactam tolerance in *Ab* largely relies on the formation of cell wall deficient spheroplast like structure. However, how bacteria maintain its structural integrity without cell wall is poorly understood. Here we uncovered the molecular determinants that drive meropenem tolerance in *Ab.* We showed both outer membrane integrity and peptidoglycan (PG) maintenance genes are required for maintaining bacterial fitness during meropenem insult. Notably, PG recycling plays critical role for this extended survival. Additionally, we found PG recycling promotes cell elongation in *Ab*. Together, these finding emphasizes that both outer membrane rigidity and PG recycling is vital for maintaining cell homeostasis in *Ab.*

Manuscripts Published

1. **Islam, N.**, Kazi, M.I., Kang, K.N., Biboy, J., Schargel, R.W., Boutte, C.C., Dörr, T., Vollmer, W., Boll. J.M., 2022. Peptidoglycan recycling promotes outer membrane integrity and carbapenem tolerance in *Acinetobacter baumannii*. *mBio*. 13(3):e0100122. (PMID: 35638738)

List of Figures

CHAPTER 1: INTRODUCTION

The discovery and clinical implementation of antibiotics is an important achievement that revolutionized medicine in the 20th century. Without antibiotic treatment, major surgeries, transplants, and chemotherapy would not be possible. However, our antibiotic repertoire is failing at an alarming rate. Many studies have shown that antibiotic resistance is a major determinant of antibiotic treatment failure, but other phenomena such as tolerance, heteroresistance, and persistence also contribute [1]–[3]. Furthermore, recent studies have shown that heteroresistance, persistence, and tolerance are also steppingstone that promote resistance through horizontal gene transfer or genetic mutations. [4]. Antibiotic resistant pathogens are widespread in United states and across the world. According to Center for Disease Control, more than 2.8 million people are infected by antimicrobial-resistant pathogen in United States every year [5]. Together with the fact that we are not developing new antimicrobials, our pending inability to effectively treat infections is considered a global crisis.

While resistance is the major contributor of the treatment failure, understudied mechanisms including tolerance also contribute to pathogens acquiring the ability to overcome bactericidal agents [6]. Antibiotic tolerance is defined as a survival of a bacterial population for prolonged periods in the presence of an otherwise lethal antibiotic concentration [7], [8]. Prolonged survival may directly or indirectly contribute to antibiotic treatment failure. After the antibiotic is degraded or diluted, tolerant cells resume normal growth implying that tolerance directly contributes. Furthermore, extended survival also increases the probability that the susceptible cell can acquire a true resistance determinant through horizontal gene transfer, an indirect mechanism [9]. While a plethora of comprehensive studies have uncovered various Gram-negative resistance mechanisms, the molecular factors that lead antibiotic tolerance are not well-understood [10].

Beta-lactam antibiotics such as penicillin are the most prescribed antibiotics, and can treat Gram-negative bacterial infections safely and effectively [11]. Beta-lactams antibiotics bind with the conserved active-site in penicillin binding protein (PBPs) and inhibits the crosslinking between adjacent peptidoglycan (PG) [12]. PG is an essential structure for most Gram-negative bacteria. Perturbations induce rapid cell lysis and cell death [13], [14]. Carbapenems are also important beta-lactam therapeutics due their broad-spectrum activity against Gram-negative bacterial infections and the fact that they are resistant to many common resistance mechanisms [15]. Meropenem and imipenem are two clinically important carbapenem antibiotics. Both are considered the last line prescriptions against the multidrug resistant Gram-negative bacterial infections [16]. However, since *Acinetobacter baumannii* (denoted as *Ab*) is so highly drug resistant, they are first line options[17]. Accordingly, carbapenem resistant *Ab* has also emerged in recent years, and carbapenem-resistant *Ab* has been labelled a critical threat to public health according to the Center for Disease Control [18], [19]. *Ab* disease manifests as urinary tract, skin, soft tissue, bone and lung infections, primarily in immunocompromised patients and is associated with high mortality [5], [20]. *Ab* can also withstand desiccative conditions and rapidly acquire resistance to conventional antibiotics [21]. Carbapenem resistance in *Ab* is primarily due to acquisition of carbapenems enzymes that hydrolyze almost all beta-lactam antibiotics [22]. However, data herein also shows that outer membrane porins, which provide the major entryways for carbapenems, and efflux pumps also contribute to carbapenem survival during treatment. In addition to widespread carbapenem resistance, susceptible *Ab* isolates also show tolerance to lethal doses of carbapenems, which increases the probability that they will overcome treatment [23]. Despite endeavors to understand tolerance mechanisms in other Gram-negative bacteria, carbapenem tolerance in *Ab* is not fully understood.

In my graduate work, I characterized the molecular factors that led meropenem tolerance in *Ab*. I found that susceptible *Ab* isolates can survive for extended periods in high levels of meropenem, indicative of tolerance. Like other carbapenem tolerant Gram-negative organisms, treatment induces spheroplast formation. Antibiotic removal induced reversion to the canonical *Ab* coccobacillus morphology. Transcriptome sequencing of early timepoints during treatment, before spheroplast formation, showed differential expression of genes that likely coordinate a regulatory response to reduce periplasmic meropenem concentrations, including decreased porin expression and increased expression of efflux pumps. Transposon-insertion sequencing also suggested specific genes were required for bacterial survival during treatment. Notably, PG recycling genes were required for *Ab* fitness during meropenem treatment. Overall, we found *Ab* coordinates several pathways to limit meropenem induced cell envelope damage. Our findings advance our understanding of molecular factors that contribute to carbapenem treatment failure and resistance mechanisms that regulate bacterial responses in presence of antibiotics.

Furthermore, I also found that PG recycling is required for cell elongation in *Ab*, which was an unexpected finding that likely has important implications for carbapenem treatment. Cells that were unable to elongate due to PG recycling disruption showed decreased tolerance to meropenem. PG recycling includes several genes including, *elsL*, *ampD*, *nagZ* and *mpL* that are required for PG turnover products during growth and cell envelope maintenance. Mutants with defects in any single gene in the PG pathways produce spheroid cells, which appear morphologically identical to rod-dependent PG mutants such as *mreB, rodA* or *pbp2*. The morphological similarities of PG recycling genes with Rod-dependent mutants can be explained in at least two ways: mutations could indirectly induce PG defects that affect Rod system function, or PG recycling proteins could directly provide lipid II substrate to the Rod-complex to promote

axial PG synthesis. These initial studies will serve as a starting point for a project to understand how PG recycling contributes to *Ab* growth and antibiotic resistance.

Antibiotic treatment failure is a serious problem throughout healthcare settings and *Ab* is one of the most difficult-to-treat Gram-negative pathogens. Since carbapenem tolerance has recently been proposed to serve as a stepping-stone to true resistance, these studies add to the body of knowledge to understand how true resistance emerges, while also serving to characterize molecular factors that pathogens use to strategically survive antibiotic treatment. Together, these studies unraveled links between tolerance and resistance evolution, which could be used for development of new therapeutic options to slow the spread of antibiotic resistance in *Ab* and other clinically significant Gram-negative pathogens.

CHAPTER 2: LITERATURE REVIEW

DISCOVERY OF BETA-LACTAM ANTIBIOTICS AND THEIR CLINICAL USE: The concept of using chemicals to cure diseases was first introduced in the Ancient Egypt, Babylon, the Far East, and Incas. More recently antimicrobial compounds produced by microorganisms were discovered. In 1871, Sir John Burdon-Sanderson observed *Penicillium* mold was able to reduce the turbidity of the media exposed to air [24]. During that time William Roberts, John Tyndall and Joseph Lister also observed the same phenomena and anticipated its antimicrobial activity [25]. The most notable antibiotic discovery was by Scottish bacteriologist Alexander Fleming, who inoculated his own nasal secretion onto an agar plate to visualize growth of his nasal flora. Surprisingly, there was no colony growth. Initially, Fleming hypothesized there may be some diffusible substance present in the nasal sample that affected the bacterial growth. In 1928, Fleming was studying the relationship of *Staphylococcus* and its virulence based on their colony morphology. After an experiment, he stacked the plates on the corner of the laboratory bench and left for vacation. When he returned, he discovered some mold grew near the bacterial colonies. The colonies directly surrounded by the mold looked transparent, indicative of lysis. This observation led him to realize that mold has microbial antagonistic property [2], [26]. After more than a decade, in 1939, Howard Walter Florey, head of the Sir William Dunn school of Pathology, Oxford University, England and his recruited biochemist Ernst Chain reported that Fleming's penicillin useful to cure infections in mice, rats and cats. After one year, *penicillin* is first used in 10 human beings who were suffering from S*taphylococcus* infections[i]. During 1942-1943, *penicillin* was produced in United states and England in a large scale for clinical uses and during World War II in 1945, benzylpenicillin was used in the treatment of allied soldiers. In 1946,

Fleming, Florey and Chain were awarded to the Noble prize for medicine for their groundbreaking discovery of penicillin [27].

The work by the Florey's group also revealed the production of different penicillin compounds based on different strains, culture conditions and media. However, they all contain a four membered beta-lactam rings [28]. Following the discovery of penicillin, the other natural beta-lactam has been discovered such as cephalosporins, monobactams, carbacephems and carbapenems (**Figure 1**) [29]. The penicillin contains a nucleus of 6-animopenicillanic acid (lactam plus thiazolidine) ring and other ringside chains. Cephalosporin beta-lactams are historically used for treatment against Gram-positive bacteria. They contain a 7-aminocephalosporanic acid nucleus and side-chain containing 3,6-dihydro-2 H-1,3-thiazane rings [30]. Monobactams are monocyclic and effectively used against aerobic Gram-negative bacteria. In contrast to other beta-lactam antibiotics, the beta-lactam ring on monobactams is not fused with another ring. Carbapenems are a class of beta-lactam antibiotics effective for severe, high-risk bacterial infections. Their defining structure is a carbapenem coupled to a beta-lactam ring that confers protection against most of the Gram-negative pathogens. Carbacephems has the similar structure of cephalosporins except sulfur is substituted by the carbon (**Figure 1).**

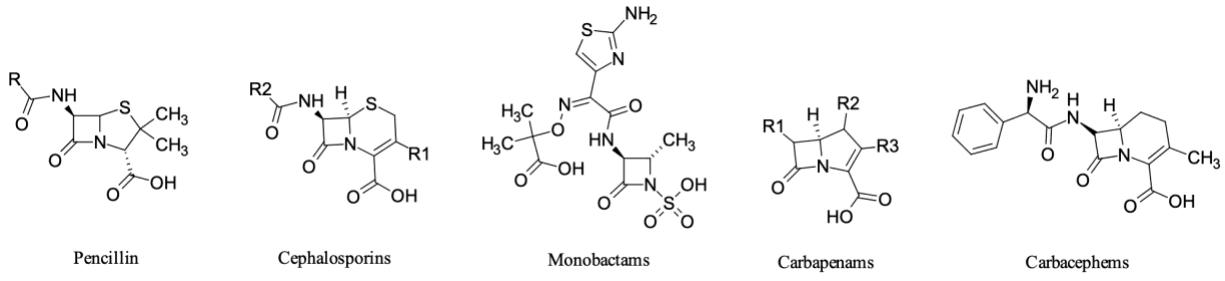

Figure 1: Structure of penicillin and Its derivatives as described in Chen *et al* (2021)[29]

BETA-LACTAM'S MECHANISMS OF ACTION:

β-Lactam antibiotics are the most widely used antibiotic to treat Gram-negative bacterial infections due to their safety and efficacy[31]. In the United States, β-Lactams account for 65% of prescriptions[32]. Tipper and Strominger first identified the antibacterial activity of β-lactams. The small molecules bind and inhibit the transpeptidase activity of penicillin-binding proteins that crosslink adjacent PG strands. These proteins are essential components of bacteria that build and maintain PG, which protects the cell from high internal osmotic pressure. PG is composed of the repetitive glycan strands and crosslinked by the peptide chains. The enzyme catalyzes the crosslinking of the peptides during PG synthesis known as the penicillin-binding proteins (PBPs) [33], [34]. Bacteria produce several PBPs which varies in terms of catalytic domains. PBPs with glycosyltransferase domains catalyze glycosidic linkages between existing PG and lipid II. Next, the transpeptidase domain catalases the formation of covalent cross-links between peptide chains on adjacent strands. The transpeptidase domain activates with the nucleophilic attack of an active-site serine residue onto a PG pentapeptide chain at the amide bond connects the D-Ala, D-ala amino acid residues and forms a covalent complex where a transpeptidase serine is acylated with a tetrapeptide. Then a nucleophilic side chain on the third amino acid (L-lysin, meso-diaminopimelic acid) of another pentapeptide attacks the ester carbonyl of the peptide–PBP complex and create a covalent bond between called 4 → 3 peptide cross-link in which the fourth residue of one peptide chain is covalently attached to the third residue of another peptide. This crosslinking between the adjacent strands creates a cage-like layer and forms the PG lattice, a load-bearing structural support that protects the cell from the internal turgor [12]. beta-lactam antibiotics covalently inhibit the transpeptidase domains of PBPs and disrupts PG cross-linking [12]. beta-lactams have structural similarities with the D-Ala, D-Ala residues in pentapeptides and it forms

an inhibitory complex with the transpeptidase serine that resembles the first step of the transpeptidase mechanism which results in inhibiting the cell wall synthesis and results in cell death [35]. However, bacteria use several mechanisms to evade the beta-lactam targets such as production of beta-lactamases, efflux pump expression, and decreased porin expression, which also contribute to beta-lactam treatment failure.

CARBAPENEM BETA-LACTAMS: Carbapenems are considered the most potent broad-spectrum beta-lactams against Gram-negative bacterial infections [36]. Carbapenems are used in monotherapy during serious conditions such as intra-abdominal infections and to treat neutropenic patients[32]. Carbapenems typically bind multiple penicillin binding proteins in Gram-negative bacteria, including PBP1a, 1b, 2 and 3. They are well tolerated and have stability to most beta-lactamases[32]. Meropenem and imipenem are two carbapenems prescribed as a last-line antibiotics against MDR Gram-negative pathogens, a broad spectrum of antimicrobial activity that exceeds that of most other antimicrobial classes [37]. However, in some cases, it is reported than meropenem is more effective than imipenem. For example, meropenem shows two to 16-fold more active than imipenem against Gram-negative aerobes, Enterobacteriaceae, *Haemophilus influenzae* and *Neisseria gonorrhoeae* [38]. Carbapenems are structurally similar to penicillin, and are bicyclic, consisting of the characteristic four-member beta-lactam ring fused to a five-membered pyrroline ring containing a carbon instead of the sulfur at the C1 position and a double bond between C2 and C3 positions (**Figure 1**). Modification of the groups around the beta-lactam ring allow carbapenems to possess potent broad-spectrum activity and effectively resist many common resistance mechanisms [12]. Despite efficacy to treat multidrug resistant bacterial infections, carbapenem resistance mechanisms have emerged.

ANTIBIOTIC TREATMENT FAILURE: Undoubtedly antibiotics are a key development to promote many advances in modern medicine. The term antibiotic was first used by Selman Waksman in 1941 to describe any small molecule made by the microbe that antagonizes the growth of the other microbes [39]. After the development of penicillin in 1940-1945, the discovery of several other antibiotics such as streptomycin, chloramphenicol, tetracycline produced from soil bacteria was one of the most successful form of chemotherapy in the history of medicine [40]. However, the failure of antibiotic treatment has become a serious issue around the world. Every year nearly 5 million deaths worldwide due to antimicrobial resistant (AMR) infections. More recently, the AMR crisis is known as the slow-motion pandemic with estimated 10 million deaths/year by 2050 [41],[40]. There is a variation of the AMR patterns across the world and different countries experience different major problems. For example, in European countries the rate of *Staphylococcus aureus* infection caused by methicillin-resistant strains (MRSA) is ~10% of the bloodstream, while in other countries resistance rate is closer to 50%. Due to overuse of antibiotics, microbes adjust their surroundings and modify themselves so that they can replicate, survive, and spread in the presence of stress or antibiotics. Antibiotics overuse and abuse, inexact diagnosis and improper antibiotic prescribing, patient sensitivity, loss and self-medication, poor personal hygiene and widespread agricultural use are the potential reasons of evolving resistant populations and thus contributes to the treatment failure [42].

ANTIBIOTIC RESISTANCE AND REASONS BEHIND IT: Bacteria acquired a remarkable plasticity in respond to a wide range of environmental stress including the presence of antimicrobials and causes the failure of the treatment of antibiotics. The World Health Organization (WHO) declared antibiotic resistance is one of the three most important public health crisis of the 21st century [43]. According to the Center for Disease Control and Prevention, at least

23,000 people in the USA die annually due to antibiotic resistant bacterial infections [44]. Antibiotic resistance is defined as the ability of bacterial populations to grow in the presence of bactericidal drugs, and it is quantified by an increase in minimum inhibitory concentrations (MIC). Generally, bacteria use two genetic strategies to escape the antibiotic attack. 1) Mutation of a gene(s), which is often associated with the mechanisms of action or 2) horizontal gene transfer (HGT), which is generally acquired through uptake and expression of foreign DNA encoding a resistance mechanism [45]. For mutational resistance, a susceptible population develops mutation(s) in genes that inhibits the antimicrobial activity, which promotes bacterial survival. Once a resistant mutant emerges, the susceptible population is eliminated by the antibiotics, and selection drives survival of the resistant bacterial cells. Mutations alter the antibiotic actions via one of the following mechanisms: i) Modification of antimicrobial target which is acquired by the destruction of antibiotics or by the chemical alternations of the antibiotics; ii) a decrease in the drug uptake by several outer membrane porins, for example, in *Pseudomonas aeruginosa*, mutation in *OprD* genes reduces the uptake of beta-lactam antibiotics and promotes bacterial survival; iii) activation of efflux pumps that expel antibiotics out of the cell; and iv) global changes in the metabolic pathway via modulation of regulatory network (**Figure 2**) [45], [46]. Gram-negative bacteria mostly use all four mechanisms, while Gram-positive bacteria rarely use the uptake of drugs and drug efflux mechanisms due to the absence of the outer membrane [42], [45]. Beside mutations, horizontal gene transfer also drives antimicrobial resistance. Bacteria share genetic information upon their own environment niche. There are three main strategies by which bacteria can exchange their genetic information such as, i) transformation (incorporation of the naked DNA), this is the simplest type of HGT where bacteria able to incorporate naked DNA to develop resistance; ii) transduction (Phage mediated)[47] and iii) conjugation (also referred as

'bacteria sex'). Conjugation is a very efficient method of gene transfer that involves cell to cell contact where bacteria use mobile genetic elements (MGRs) as a vehicle to share valuable information. The common mobile genetic elements are plasmids and transposons [48]. It is reported that MGRs play a crucial role in disseminating the antimicrobial resistance among clinical isolates. Overall, antibiotic resistance is a global health crisis, and it is a well-defined cause of antibiotic treatment failure. However, very little is known about another potential phenomena of the treatment failure called antibiotic tolerance [49], [8]. Antibiotic tolerance can directly or indirectly contribute to treatment failure. Despite several studies focused on antibiotic tolerance and how it contributes to treatment failure, this remains understudied and underappreciated [50].

.

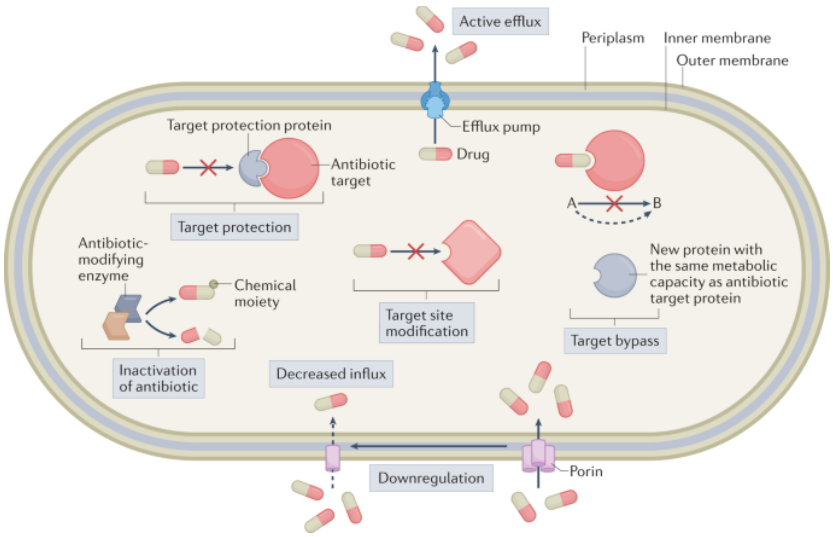

Figure 2: Molecular mechanisms of antibiotic resistance as described by Darby *et al*.,2022 [46].

Gram-negative bacterial species can evade the antimicrobial therapy by decreasing influx through reduced permeability, increasing efflux of toxic compound out of the cell, enzymatically inactivating the drug, or modifying the antibiotic target site.

ANTIBIOTIC TOLERANCE: Antibiotic tolerance is another phenomenon that also leads to antibiotic treatment failure. However, the underlying are poorly defined. Tolerance is defined as the ability of susceptible bacterial population to remain viable for extended periods after exposure of bactericidal drugs [49], [50]. Unlike resistance, it cannot be quantified by minimum inhibitory concentrations (MIC) or by measuring the zone of inhibition around an antibiotic containing disk [50]. The tolerant cells typically have a prolonged lag phase or shorten exponential phase, where cells are not actively growing in the presence of bactericidal antibiotics [51]. In the presence of the antibiotic action, tolerant cells slow down their metabolism so that they prevent lethal damage caused by the antibiotic. Tolerance is considered as the steppingstone in acquisition of true resistance. Previous studies show that in *E. coli* evolution of resistance is facilitated by the early appearance of tolerance mutations [1]. The idea that tolerance leads to true resistance was first established in 1980s, which is validated *in vitro* in a diverse assortment of microorganisms and a diversity of antibiotics. Another study showed that MRSA bacteremia in which antibiotic tolerance facilitated the development of resistance to three anti-staphylococcal therapies over a six-week clinical course [52]. In some Gram-negative bacterial species for example, in Enterobacteriaceae, spheroplast production has been reported after exposure to the lethal level of cell wall synthesis inhibitors and recovery from spheroplast state requires cell envelope stress responses, cell wall synthesis functions and a reduction in the formation of ROS [53]. In *Enterobacter cloacae*, the PhoPQ two component system regulates modification of the lipid A domain of lipopolysaccharide to promote carbapenem tolerance [54]. Overall, these studies provide clear evidence that tolerance facilitates the development of true resistance. However, the molecular mechanism of tolerance is understudied and not well characterized [8].

Ab AND ITS PATHOGENICITY: Since the multidrug-resistant (MDR) pathogen has become a serious concern throughout the world, he World Health Organization (WHO) declared antimicrobial resistance is one of the three most important problems in human health and they listed the most common and serious MDR pathogens named as **ESKAPE** standing for ***E****nterococcus faecium*, ***S****taphylococcus aureus*, ***K****lebsiella pneumoniae*, ***Ab***, ***P****seudomonas aeruginosa*, *Enterobacter* spp. *E. faecium* is a Gram-positive colonize in the gastrointestinal tract and lead several diseases such as endocarditis and neonatal meningitis. *S. aureus* is also a Gram-positive colonize in skin and upper respiratory tract and involved in skin infections and pneumonia. *K. pneumoniae* is a Gram-negative involved in respiratory and urinary tract infections. *P. aeruginosa* is a ubiquitous Gram-negative that causes several infections including respiratory, urinary, and skin infections and *Ab* is a Gram-negative causes urinary and respiratory diseases[55].

Among them, CDC listed carbapenem resistant *Ab* is one of the most urgent public threat because it is resistant to nearly all conventional antibiotics and cause deadly infections, typically in immunocompromised patients[18]. *Ab* is a Gram-negative, aerobic, pleomorphic, and non - motile opportunist pathogen primarily associated with hospital acquired infections. Most notably, it causes ventilator-associated pneumonia, blood steam infections, urinary tract infections and meningitis [56]. It colonizes under the skin and respiratory and oropharynx secretions of the infected individual, and it is designated as a “red alert” superbug [57]. *Ab* has gained been gained attention as a “Iraqibacter” since its rapid spread among the US military during the war of Middle Eastern in Iran, Kuwait, and Afghanistan in 2002-2004[58]. Several virulence factors have been reported during infections caused by *Ab* such as outer membrane porins, phospholipases, proteases, lipopolysaccharide, capsular polysaccharides, protein secretion systems, and iron-chelating systems. *Ab* possess a remarkable capacity to survive in the desiccative conditions and

interestingly some of the species can stay alive in the dry condition for almost 100 days [59]. It is reported that the presence of capsular polysaccharide promotes *Ab* to survive during desiccation. These polysaccharides composed of repeating carbohydrate units and function as a glycan shield that encompasses the bacterium and protects them from external threat. It can also form robust biofilm during skin and soft-tissue infections. Furthermore, this pathogen form biofilm on most abiotic surfaces including health-care-associated equipment such as endotracheal tubes and polycarbonate and stainless steels. Also, *Ab has* hypermotility or surface associated motility on semi solid surfaces. Barker and Maxted in 1975 first observed this type of motility and they named it 'swarming' motility and this surface motility is associated with increased virulence. Some of the recent studies showed that *Ab* surface associated motility is relying on the synthesis of 1,3-diaminopropane, quorum sensing and LOS production [59]. Carbapenem antibiotics meropenem and Imipenem considered the last line treatment to treat multidrug resistant Gram-negative bacterial infections, while they are considered the first resort prescription to treat *Ab infections* [60]. It is thought that every year around 1 million people worldwide affected by *Ab* infections and 50% of them reported as carbapenem resistance. They pose several mechanisms to evade carbapenem antibiotics such as lack or small size of porins, expression of efflux pump, expression of beta-lactamases[61]. Most carbapenem resistant *Ab* isolates are called extensively drug resistance and some are even resistant to last resort antibiotic colistin [62].

THE GRAM-NEGATIVE CELL ENVELOPE: The bacterial cell envelope is a complex structure that surround and protect the cytoplasm from unpredictable dilute and hostile environment. To survive and protect themselves bacteria have evolved a complex cell envelope that protects them but allows selective passage of nutrients from outside environment to inside the cell. In Gram-negative bacteria, the cell envelope is a complex multilayered structure consists of three distinct

layers in the envelope: The asymmetric outer membrane, the PG cell wall and the cytoplasmic inner membrane (**Figure 3**) [63]. Each envelope layer is essential for viability. The outer membrane serves as a permeability barrier that restricts the entry of toxic compounds, the PG cell wall provides cell shape and osmotic protection to the cell and the Inner membrane delimits the cytoplasm and hosts many vital cellular processes including respiratory systems [64]. The outer membrane of the Gram-negative bacteria is a unique and distinguishing feature which is absent in Gram-positive bacteria. The outer leaflet of the outer membrane is composed of glycolipids; principally the lipopolysaccharides (LPS); whereas the inner leaflet of the outer membrane composed of phospholipids [65]. LPS molecules works as a barrier against small, hydrophobic molecules making bacteria resistant to many antimicrobial compounds. In addition to this, LPS plays a crucial role in bacteria-host interactions. It plays an important role in bacterial pathogenicity due to the endotoxic shock modulated by the host immune system [66]. It is a glucosamine disaccharide with six or seven acyl chains, a polysaccharide core and an extended polysaccharide chain called the O-antigen. Typically, the outer membrane proteins can be divided into two classes, lipoproteins and ß-barrel proteins. Lipoproteins contain lipid moieties that are attached to an amino-terminal cysteine residue. In *E. coli*, 100 lipoproteins have been reported but the function is not known, whereas the ß-barrel proteins play essential role in cargo transport and signaling and important for membrane biogenesis (**Figure 3**) [67], [68]. Beside the outer membrane, the Inner membrane (IM) is a phospholipid bilayer; it basically works in energy production, lipid biosynthesis, protein secretion and transport. The IM and outer membrane is separated by the periplasmic space that contains the PG cell wall. PG is the major structural polymer. In most of the bacteria, the PG cell wall consists of glycan chains of repeating N-acetylglucosamine and N-acetylmuramic acids cross linked via peptide side. In most of the Gram-

negative bacteria has a thin monolayered PG whereas in Gram-positive bacteria possess a thick multilayered PG [13], [69].

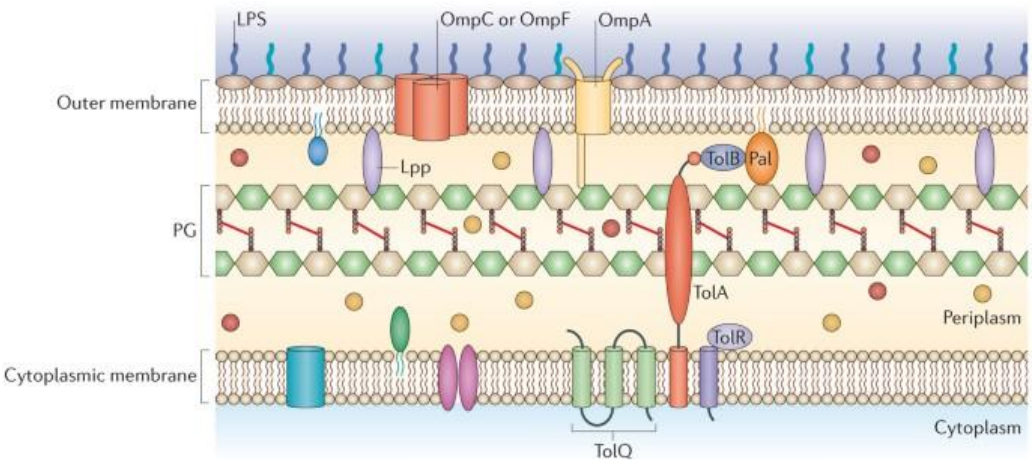

Figure 3: The Gram-negative cell structure as described in Schwechheimer and Kuehn; 2015[69].

REGULATION OF PG SYNTHESIS AND REMODELING: Bacterial cell membrane surrounded with a net-like structure called PG sacculus, which is important for maintaining cell shape and protects the cell from bursting. Multiple protein complexes such as elongasome and divisiome protein complexes are working together to build this sacculus. These complexes comprise several PG synthases, their regulators, PG hydrolases that are essential for breaking bonds within the wall to synthesize new material. The growth of the sacculus requires synthesis of new PG and its incorporation into the existing layers.

Lipid II is called the PG precursor synthesized at the inner leaflet of the cytoplasm and flipped to the periplasm by the enzyme MurJ; and utilized by the PG synthases. The PG glycosyl transferases (GTases) polymerize the glycan chains whereas transpeptidases (TPases) form peptide crosslinks. Class A penicillin-binding proteins (aPBPs) such as PBP1a, 1b, and 1c; are bifunctional (GTase/TPase); Whereas class B PBPs (bPBPs) such as PBP2 and PBP3 are monofunctional

TPases (**Figure 4**). There are two types of monofunctional GTases called SEDS (shape, elongation, division, and sporulation) proteins and monofunctional GT51 GTases. Most of the study showed that in *E. coli* or other rod shape bacterium, RodA and FtsW works as a SEDS protein which are essential for sacculus growth during elongation and division, respectively. Generally, in rod shaped bacteria there are two machineries, the elongasome (Rod complex) which is organized by the actin homologue MreB, that facilitates the insertion of new material during growth along the lateral part of the cylinder. The divisome (FtsZ complex) is maintained by the tubulin homologue FtsZ, that facilitates PG synthesis during division. MreC is essential for rod shape, and it is a widely conserved elongasome protein. MreC interacts with MreB and PBP2. PBP2 TPase stimulates the GTase activity of PBP1A and involve the attachment of newly synthesized material to a PG sacculus and these two synthases function together to synthesize and attach new PG. Whereas PBP1A localizes predominantly to the cell periphery during growth and interacts with the class B PBP2, which is essential for elongation. Whereas during division, PBP1B localizes at the mid cell and interacts with the divisome proteins such as ZipA, FtsW, FtsN, FtsQ-FtsL-FtsB and class B PBP3 [70](**Figure 4**). In *E. coli* the role of PBP1A and PBP1B is semi or partially redundant[71].

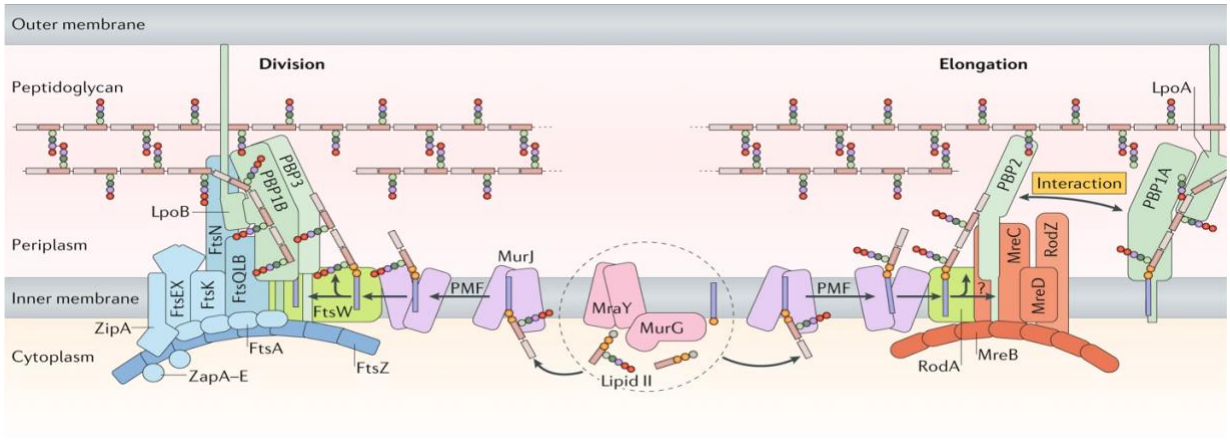

Figure 4: Schematic representation of division and elongation complex in *E. coli* during PG synthesis as described by Egan *et al*.,2022.

Along with PG synthases, PG hydrolases plays a crucial role in cleaving the bonds in PG chain and side chain branches and responsible for maintaining overall PG turnover [72]. These enzymes are also known as autolysin [73]. In Gram-negative bacteria, the fragments are released by the autolysin are transported into the cytoplasm for recycling to build ***de novo*** PG. Autolysin includes glycan cleaving lytic transglycolases, peptide-cleaving carboxypeptidases, endopeptidases and *N*-acetylmuramyl-L-alanine amidases [73]. In *E. coli* the low molecular weight (LMW) PBPs such as PBP4, PBP5, PBP6, DacD, and PBP7 are involved in endopeptidase (hydrolyzed the crosslink between mDAP of one peptide stem and D-Ala residue of another peptide stem) and D, D (cleaves the bond between D amino acids) or L, D (cleaves the peptide bond between D and L amino acids) -carboxypeptidase activity. The endopeptidase activity has space-making autolytic function that expands the sacculus during cell elongation. Without endopeptidase activity PG synthesis results in a thicker cell wall and results in integrity failure and lysis [74]. While the lytic transglycolases (LT) cleaves the glycosidic linkages between disaccharide subunits within PG strands and produced anhydro-MurNAc (anhMurNAc). In *E. coli* seven LTs are designated named MltA, MltB, MltC, MltD, MltE, MltF, and Slt70; the first six are

membrane bound while Slt70 are soluble LTs [75]. It is reported in some Gram-negative nosocomial pathogen such as *Bordatella pertussis*, these anhMurNAc released by LT activity play a role in bacterial virulence [76]. LT reactions products are either utilized for recycling activity [74], [77] or released to the surroundings to mitigate bacterial load bearing capacities [78].

PG recycling starts typically when the LT released the anhMurNAc into the environment and it is imported into the cytoplasm by an inner membrane permease, AmpG, and broken into constituent sugar and peptide by the amidase, AmpD, ß-hexoaminidase, NagZ, and LD-carboxypeptidase, LdcA (**Figure 5**) [79]. Together these reactions yield, NAG, anhNAM and free tripeptides in the cytoplasm [14] which serve as an energy source during stress conditions. Next, MpL ligates tripeptides to UDP-MurNac, which is an intermediate in the de novo PG synthesis pathway. Alternatively, in the presence of beta-lactam antibiotics, some Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Enterobacter cloacae* induce expression of AmpC beta-lactamase. These findings suggest that accumulation of 1,6 anhMurNAC by increased LT activity is sensed, and induces a response to beta-lactam activity by induction of the AmpC beta-lactamase to promote antibiotic resistance [80].

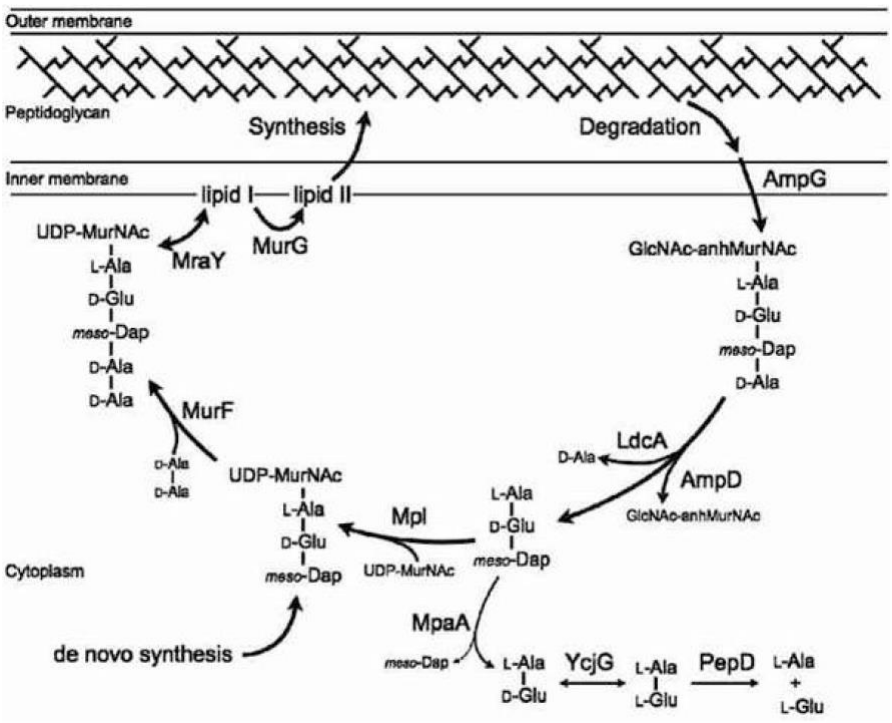

Figure 5: Schematic representation of PG recycling pathway as described by Uehara and Park,2008[81]**.**

TRANSPOSON INSERTION SEQUENCING (Tn-seq): Transposon insertion sequencing (Tn-seq) is used to estimate the essentiality or fitness contribution of each genetic features in a bacterial genome simultaneously [82], [83]. It combines the large-scale transposon mutagenesis along with massive parallel sequencing. The main goal is to build a mutant library of bacteria where each cells carries a single transposon insertion in the genome. This method not only detect the minor changes in genes required for mutant fitness but also precise enough to detect intergenic regions, promoter regions and essential protein domains with coding regions [83]. Tn-seq includes generation of saturating gene disruption library through transposon mutagenesis, pooling of the individual insertion mutants, extraction of genomic DNA (gDNA) from the pooled mutants and amplification and high throughput sequencing of the transposon insertion junctions [82]. The

mariner family transposon is useful for generating saturating mutant libraries and diverse bacterial background. This technique is generally plasmid encoded and can easily be modified by replacing the transposase promoter and antibiotic selection marker to be applicable in diverse bacterial backgrounds. In my current work, we have used this Tn-seq screening to understand the fitness factors required for carbapenem tolerance in *Ab.*

BETA-LACTAMASES: Production of beta-lactamase enzymes is the one of the key mechanisms of Gram-negative bacteria to emerge and dissemination of resistance. Beta-lactamase degrade the drug activity by irreversibly opening the beta-lactam ring by hydrolyzing the amide bond. The first beta-lactamase that hydrolyze penicillin was reported in *E. coli* nearly 70 years ago [84]. Over the time, with the explosion of the sequence information, there are over 4300 beta-lactamase enzymes have been characterized [85], [86]. Based on the structural and sequence similarities, beta-lactamase have been classified into four classes (A, B, C, and D) [87]. All classes are widely distributed among nosocomial Gram-negative pathogens, including *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *Ab.*

Class A, C and D enzymes are called serine-beta-lactamases (SßLs) because they require an active-site serine residue to catalyze beta-lactam hydrolysis. SßLs employ an acylation-deacylation mechanism where the nucleophilic serine attacks the carbonyl carbon of the beta-lactam and hydrolyze the amide bond and generated acyl enzyme intermediate via a tetrahedral oxyanion transition state [86]. Whereas class B beta-lactamases rely on one or two active site Zn^{2+} ions to inactivate beta-lactam antibiotics and are therefore referred to as the metallo-beta-lactamases (MßLs). The most notable class B MßLs include imipenemase (IMP), Verona integron-encoded metallo-beta-lactamase (VIM), Sao Paulo metallo-beta-lactamase (SPM), Germany imipenemase (GIM), New Delhi metallo-beta-lactamase (NDM), Florence imipenemase

(FIM) [88], [89]. Class D enzymes are all termed oxacillinase (OXA); particular concern surrounds the OXA-23 and 24/40, and the OXA-48 groups, responsible for carbapenem resistance in *Ab* and Enterobacteriaceae, respectively.

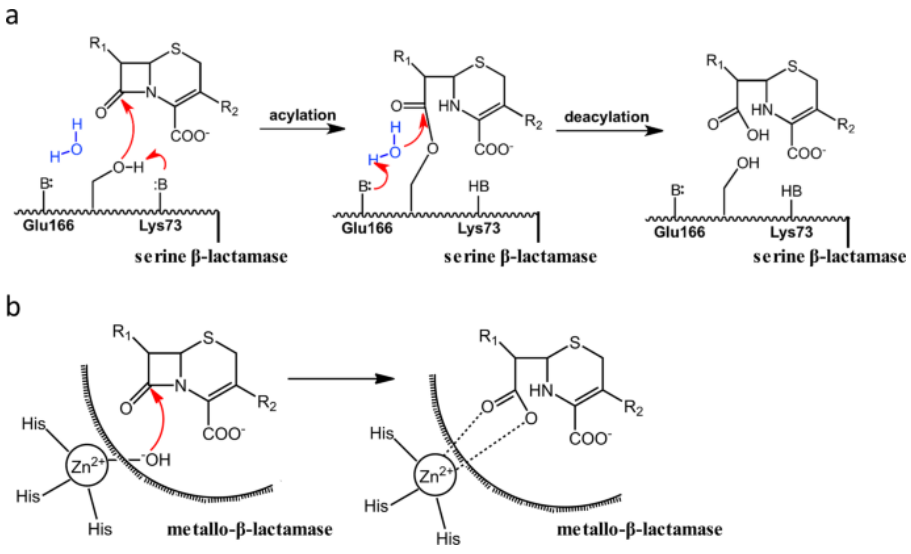

Figure 6: The catalytic mechanisms of major beta-lactamases as described by He *et al.*, 2020[90] a) showing the catalytic activity of serine-based beta-lactamases that employ a two-step process; b) showing the mechanisms of class B metallo-beta-lactamases.

LIPID A MODIFICATIONS: Due to the complex structure of the outer membrane, antibiotic treatment has been increasingly challenging. The outer membrane is an asymmetric bilayer, with the inner leaflet composed of glycerophospholipids and the outer leaflet composed of LPS. LPS has structural role in decreasing permeability and increasing rigidity of the cell. It also called the most potent stimulators of the host innate immune system [91]. The LPS is consists of structurally and biologically three distinct domains: 1) The acylated lipid A that forms the outer monolayer of the outer membrane; 2) The core oligosaccharide linked with lipid A and 3) O-antigen. LPS with

all three domains are called smooth-form LPS, while LPS without O-antigen are named rough-form LPS or lipooligosaccharide (LOS) [91].

The lipid A is the toxic component of lipopolysaccharide that is made up of $\beta(1 \rightarrow 6)$-linked glucosamine disaccharide backbone which is mostly phosphorylated at position 1 and 4′ of the saccharides and acylated at positions 2 and 3 of each monosaccharide portion [92]. The lipid A biosynthesis starts in the cytoplasm with the formation of Kdo_2-lipid A. Kdo stands for 3-deoxy-D-manno-octulosonic acid or endotoxin. Nine conserved enzymes working consecutively and produce hexa-acylated lipid A (**Figure 7)** [93]. The first seven enzymes produce the precursor Kdo_2-lipid IV_A; while the last two enzymes LpxL and LpxM completed the synthesis of hexa-acylated lipid A.

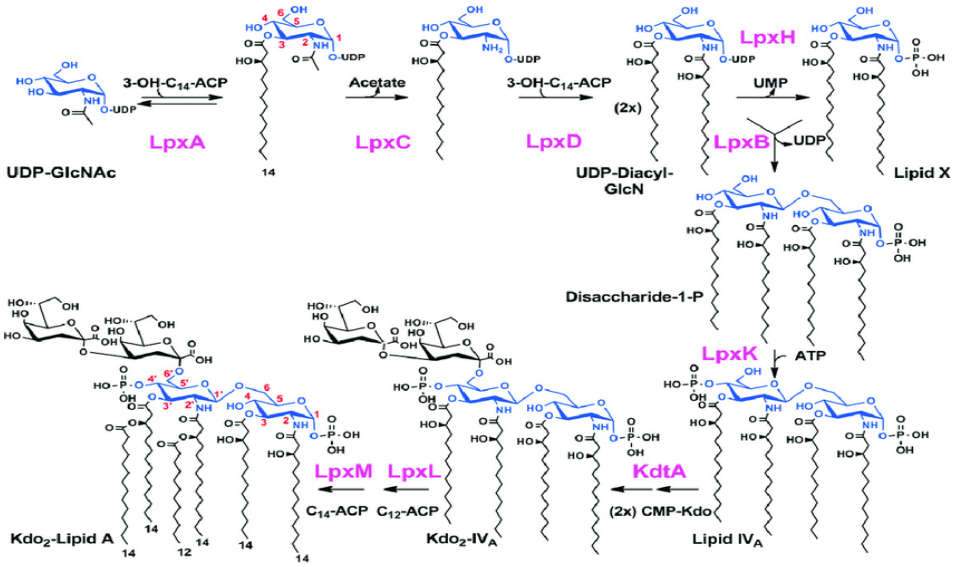

Figure 7: Schematic representation of Lipid A biosynthesis by Raetz *et al.* Nine consecutive enzymes working together to build hexa-acylated lipid A[94].

LpxL catalyzes transfer of laurate (C12:0) followed by LpxM-dependent myristate (C14:0) addition in a stepwise manner to complete synthesis of the hexa-acylated and bis-phosphorylated

Kdo2-lipid A molecule. However, many Gram-negative pathogens alter their lipid-A structure for surviving into the host by alter their outer membrane permeability, to evade host immune detection and promotes antibiotic resistance. One of the most common way bacteria modified their lipid A by the production of hepta-acylated lipid A to fortify it's outer-membrane and reduce host immune recognition. In the presence of signal, the bacteria initiate a regulatory phosphorylation cascade that increases the production of the outer membrane protein PagP. PagP transfers a palmitoyl (C16:0) to the lipid A with phosphatidylethanolamine. However, the nosocomial pathogen *Ab* produces the hepta-acylated lipid A in a PagP-independent fashion where the LpxM$_{Ab}$ *(Ab* LpxM) works as a dual acyltransferase that transfers two lauroyl groups onto the lipid A and produce hepta-acylated lipid A [21]. These hepta-acylation is unique in *Ab* and it helps the bacteria to survive in desiccative conditions and increases the resistance to polymyxin CAMP (Cationic antimicrobial peptide) such as colistin which is considered as a last-resort antibiotic agent.

OUTER MEMBRANE PORINS: The outer membrane is the unique feature of Gram-negative bacteria that serves for protections and provides nutrients for viability. It contains a several fully integrated membrane proteins and provides essential function for the cell such as nutrient uptake, cell adhesion, cell signaling and waste export. In the pathogenic strains, some outer membrane proteins (OMPs) serve as a virulence factor such as nutrient scavenging and evasion of the host defense mechanisms [68]. The OMPs are also called β-barrel proteins and has 8 to 26 strands. The short loops are on the periplasmic side, while the large loops are between the strains on the extracellular side. Due to this characteristic, these OMPs have a high membrane stability and they are capable to fight against extremely harsh environment. Different types of OMPs have different functions. In *Ab* the main outer membrane proteins included BamA, LptD, Omp33-36, OmpW, CarO, OmpA and OprD. CarO and OprD involved with carbapenem resistance[ii]. LptD mediates

the transportation of LPS to the outer membrane; OmpW is involved in modulating homeostasis of iron ion in bacteria [95]. Omp33-36 is a channel for the passage of water, which can induce apoptosis of host cells by activating caspase and regulate autophagy. Among those OMPs, OmpA is known for its virulence factors. It regulates the adhesion, aggressiveness, and biofilm production. OmpA was first identified in 1974 in *Escherichia coli* (*E. coli)* as a heat-modifiable protein and it is originally purified in 1977. The molecular mass ranges is from 28 KDa to 36 KDa. It is the surface exposed OMP and most abundant proteins in Gram-negative bacteria [96]. While locating in the outer membrane OmpA is non covalently bind to the diaminopimelate acid of the PG by its C-terminal domain. Overall, these data suggests that OmpA plays a key role in maintaining bacterial surface integrity and have a role in PG maintenance. OMP also participates in extrusion of antimicrobial compounds from periplasmic region and coupled with the efflux system in inner membrane and that's how involved in antibiotic resistance [97].

EFFLUX PUMPS AND IT'S FUNCTIONS: Extruding the drug actively from the cell against their gradients to reduce the cytosolic concentration of antimicrobials is one of main reasons of antibiotic resistance and it is mediated by several integral membrane transport proteins called efflux pump [98].There are seven families or superfamilies of transport proteins has been reported including **ABC**, ATP-binding cassette superfamily; **MFS**, major facilitator superfamily; RND, resistance-nodulation-cell-division superfamily; **MATE**, multidrug and toxic compound extrusion family; **DMT**, drug/metabolite transporter superfamily; **PACE**, proteobacterial antimicrobial compound efflux family; **AbgT**, *p*- aminobenzyoyl-glutamate transporter family (**Figure 8**). These distinct groups of efflux pumps can move substrates across the outer membrane. A variety of transporter proteins that is expressed in the inner membranes that use metabolic energy to concentrate metabolites in the cytoplasm and contributes the requirement of PG to prevent osmotic

lysis. Some efflux pumps contain single protein component transporters that export substrate from the cytoplasm to periplasm or outer leaflets of the inner membrane. Whereas other efflux systems are composed of multiple subunits such as tripartite complexes [99], where an integral inner-membrane protein associates with periplasmic and outer-membrane proteins to move substrates across the outer membrane. These type of efflux systems are very effective to export the drugs such as beta-lactams from the periplasm. In *E. coli* the co-expression of tripartite pump with a single component transporter shows multidrug resistance.

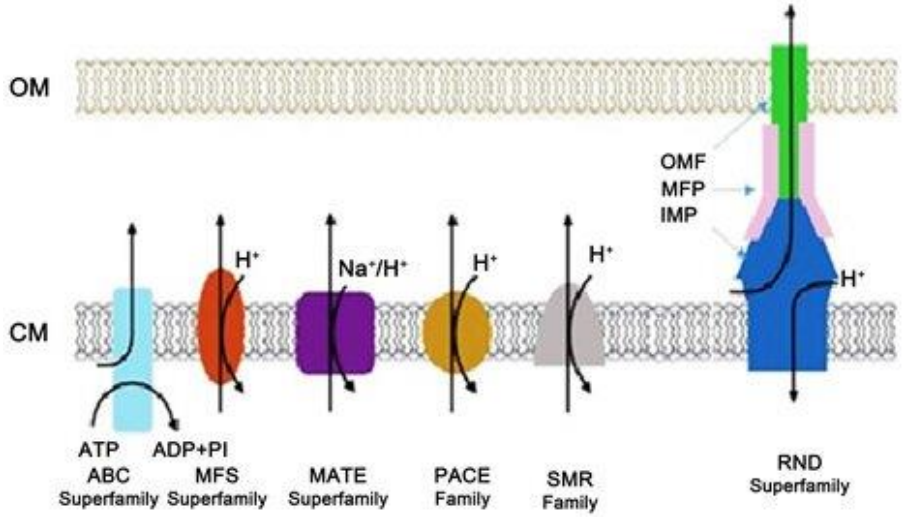

Figure 8: Structure of major *Ab* efflux pumps[100].

Ab displays high levels of multidrug resistance to a broad range of antimicrobial agents. It contains three RND-family efflux systems including AdeABC, AdeFGH and AdeIJK (**Figure 8**); two MFS system pumps and one membrane each of MATE and SMR families of efflux systems that pump out a wide range of antimicrobial agents from the periplasm. Among the RND efflux system, the AdeABC is known to increase aminoglycoside resistance; whereas AdeIJK system in *Ab*, is known to pump out a broad range of antibiotics includes beta-lactams, chloramphenicol,

tetracyclines, and erythromycin [101], [100], [102]. However, a very little is known about AdeFGH efflux system and how it contributes to antibiotic resistance.

CHAPTER 3

PEPTIDOGLYCAN RECYCLING PROMOTES OUTER MEMBRANE INTEGRITY AND CARBAPENEM TOLERANCE IN *ACINETOBACTER BAUMANNII*

Nowrosh Islam[*1], Misha I. Kazi[*1], Katie N. Kang[1], Jacob Biboy[2], Joe Gray[3], Feroz Ahmed[1], Richard D. Schargel[1], Cara C. Boutte[1], Tobias Dörr[4,5,6], Waldemar Vollmer[2], Joseph M. Boll[#1]

[1]Department of Biology, University of Texas Arlington, Arlington TX, 76019, US

[2]Centre for Bacterial Cell Biology, Biosciences Institute, Newcastle University, Newcastle upon Tyne, United Kingdom

[3]Biosciences Institute, Newcastle University, Newcastle upon Tyne, United Kingdom

[4]Weill Institute for Cell and Molecular Biology, Cornell University, Ithaca NY 14853, US

[5]Department of Microbiology, Cornell University, Ithaca NY, 14853, US

[6]Cornell Institute of Host-Microbe Interactions and Disease, Cornell University, Ithaca NY, 14853, US

*Contributed equally

[#]Correspondence:

Joseph M. Boll; joseph.boll@uta.edu

Published

Abstract

Beta-lactam antibiotics exploit the essentiality of the bacterial cell envelope by perturbing the PG layer, which induces rapid lysis. Many Gram-negative bacteria exhibit “tolerance”, the ability to sustain viability in the presence of bactericidal antibiotics for extended time periods. Despite several studies showing that antibiotic tolerance contributes directly to treatment failure, and is a steppingstone in acquisition of true resistance, the molecular factors that promote intrinsic tolerance are not well-understood. *Ab* is a critical-threat nosocomial pathogen notorious for its ability to rapidly develop multidrug resistance. Carbapenem beta-lactam antibiotics (i.e., meropenem) are first-line prescriptions to treat *Ab* infections but treatment failure is increasingly prevalent. Meropenem tolerance in Gram-negative pathogens is characterized by morphologically distinct populations of spheroplasts, but the impact of spheroplast formation is not fully understood. Here, we show that susceptible *Ab* clinical isolates demonstrate tolerance to high-level meropenem treatment, form spheroplasts with the antibiotic and revert to normal growth after antibiotic removal. Using transcriptomics and genetics screens, we show several genes associated with outer membrane integrity maintenance and efflux limit beta-lactam entry into the periplasm. Genes associated with PG homeostasis in the periplasm and cytoplasm also answered our screen. Specifically, we defined the enzymatic activity of PBP7 and ElsL (also known as LdtK), which are tolerance determinants in *Ab*, and disruption of these pathways also compromised the cell envelope barrier function. These data show that outer membrane integrity and PG recycling are tightly linked in their contribution to *Ab* meropenem tolerance.

Importance

Carbapenem treatment failure associated with "superbug" infections has rapidly increased in prevalence, highlighting an urgent need to develop new therapeutic strategies. Antibiotic tolerance can directly lead to treatment failure but has also been shown to promote acquisition of true resistance within a population. While some studies have addressed mechanisms that promote tolerance, factors that underlie Gram-negative bacterial survival during carbapenem treatment are not well-understood. Here, we characterized a role for PG recycling in outer membrane integrity maintenance and meropenem tolerance in *Ab*. These studies suggest that the pathogen limits antibiotic concentrations in the periplasm and highlights physiological processes that could be targeted to improve antimicrobial treatment.

Introduction

The cell envelope is a dynamic barrier composed of an inner (cytoplasmic) membrane, a periplasm that includes a thin PG layer and an outer membrane, which is a selective barrier that restricts entry of toxins and antibiotics. While the PG layer is known to protect against bursting due to the cell turgor, the outer membrane also protects against lysis when external osmotic conditions change [103]. Perturbation of the outer membrane or PG envelope layers induce lysis, but regulated responses that fortify the envelope can maintain envelope homeostasis to promote pathogen survival during stress exposure [104].

Antibiotic treatment failure is a growing threat to public health and has primarily been associated with antibiotic resistance (i.e., growth in antibiotic treatment). However, antibiotic tolerance, a population's ability to survive otherwise toxic levels of antibiotic treatment for extended periods, likely acts as a steppingstone to true resistance [105]–[107]. Antibiotic tolerance

is characterized by survival of cell populations in a non-dividing state, where the MIC does not change and cells revert to normal growth when the antibiotic is removed, degraded or diluted [108]–[110]. Molecular factors that extend survival during treatment, increase the probability of resistance-conferring mutations or horizontal gene transfer to occur [105].

Carbapenems are important beta-lactam therapeutics because they possess potent broad-spectrum activity and are not susceptible to common resistance mechanisms [111], [112]. In fact, meropenem is a last-line carbapenem antibiotic used to treat multidrug resistant Gram-negative infections [113], [114]. While meropenem treatment is typically reserved to fight multidrug resistant bacteria, it is a first-line prescription against the highly drug resistant nosocomial pathogen, *Ab* [115], [116]. Carbapenem-resistant *Ab* has become commonplace among hospital acquired infections. In 2019 the Center for Disease Control listed carbapenem-resistant *Ab* as one of the most urgent threats to public health [117], and a report by the World Health Organization prioritized the pathogen as critical for new antibiotic development [118], underscoring the severity.

We reasoned that since tolerance is a prerequisite for true resistance, tolerance factors may be widespread among meropenem susceptible *Ab* strains. Defining intrinsic tolerance factors in *Ab* may offer fundamental insight into how resistance mechanisms rapidly spread among populations and provide new targets to combat tolerant pathogens. While our understanding of resistance mechanisms that cause antibiotic treatment failure has been well-documented, tolerance factors that precede acquisition of true resistance are limited.

Here, we show that susceptible *Ab* strains, including laboratory-adapted and recent clinical isolates, survive for extended periods (>24 h) in high levels of meropenem, demonstrating widespread tolerance. Meropenem induces cell wall-deficient spheroplast formation in *Ab*, as shown in other Gram-negative pathogens [119]–[121]. After removal of the antibiotic, cells rapidly

revert to the canonical *Ab* coccobacilli morphology and resume growth. Transcriptome sequencing analysis at timepoints leading to spheroplast formation showed differential expression of genes that coordinate a regulatory response to reduce the intracellular meropenem concentration. During meropenem treatment, outer membrane integrity and permeability contribute to fitness, which we show are also impacted by defects in the PG recycling pathway. PG recycling is also a major contributor to *Ab* survival during meropenem treatment, where disruption of genes encoding periplasmic and cytoplasmic PG maintenance enzymes compromise outer membrane integrity. Lastly, we also define PBP7 (encoded by *pbpG*) and ElsL (also known as LdtK) enzymatic activities, which are tolerance determinants in *Ab.* Together, these studies show several pathways that coordinate in *Ab* to limit meropenem-induced cell envelope damage. These findings provide new targets to direct antimicrobial therapies and prevent the spread of resistance.

Materials and Methods

Bacterial strains and growth

All *Ab* strains were grown aerobically from freezer stocks on Luria-Bertani (LB) agar at 37° C. Antibiotics were used at the following concentrations unless noted otherwise: 25 mg/L kanamycin, 10 mg/L meropenem, 10 mg/L tetracycline and 75 mg/L carbenicillin.

Transposon insertion sequencing. Transposon sequencing was performed as described previously [122]. The transposon machinery vector, pJNW684, was conjugated into wild-type *Ab* strain ATCC 17978 to generate a library of ~ 400,00 mutants. The pooled transposon mutant library was then screened for survival in meropenem tolerance conditions. At 0, 3 and 6 hours following incubation at 37°C in stasis, genomic DNA (gDNA) from meropenem treated and untreated cultures was isolated, sheared and transposon junctions were amplified and sequenced.

Frequency of transposon insertions was compared between meropenem treated and untreated conditions to determine factors important for carbapenem tolerance in *Ab*.

Construction of genetic mutants. *Ab* genetic mutants were constructed as described previously using the recombination-mediated genetic engineering (recombineering) method [123]. Briefly, a kanamycin resistance cassette flanked by FLP recombination target (FRT) sites was PCR amplified from the pKD4 plasmid using primers containing 125-bp flanking regions of homology to the gene of interest. The resulting linear PCR product was then transformed via electroporation into *Ab* ATCC 17978 expressing REC_{AB} (pAT03). Transformants were recovered in Luria broth (LB) and plated on LB agar supplemented with 7.5 mg/L kanamycin. All genetic mutants were confirmed by PCR.

Following isolation of genetic mutants the pMMB67EHtet^{R}::REC_{AB} plasmid was removed as described previously [124]. The isolated mutants were grown on LB agar supplemented with 2mM nickel(II) chloride ($NiCl_2$) and replica plated on LB agar supplemented with 25 mg/L kanamycin or 10 mg/L tetracycline. Mutants that were sensitive to tetracycline and resistant to kanamycin were confirmed loss of the pMMB67EHtet^{R}::REC_{AB} plasmid. To excise chromosomal insertion of the kanamycin resistance cassette, cured mutants were transformed with pMMB67EH carrying the FLP recombinase and plated on LB agar supplemented with 5 mg/L tetracycline and 2mM Isopropyl β-d-1-thiogalactopyranoside (IPTG) to induce expression of FLP recombinase. Successful excision of the kanamycin resistance cassette was confirmed using PCR.

Construction of complementation vectors. To construct the *pbpG* complementation vector, the *pbpG* (A1S_0237) coding sequence (encoding PBP7/8) with 200-bp upstream and downstream flanking regions was amplified from *Ab* ATCC 17978 chromosomal DNA (cDNA) and cloned

into XhoI and KpnI restriction sites in the pABBRKn plasmid. The resulting pPBP7 plasmid was transformed into *Ab* ATCC 17978 *⊿pbpG* background for complementation.

The *ampD, ompA* and *lpxM* complementation vector was constructed was constructed similarly with slight alterations. The *ampD* (A1S_0045), *ompA* (A1S_2840) and *lpxM* (A1S_2609) coding sequences was amplified from *Ab* ATCC 17978 cDNA and *ampD* was cloned into BamHI and SalI restriction sites, *ompA* was cloned into KpnI and SalI restriction sites in the pMMB67EHkn^{R} plasmid. *lpxM* was cloned into KpnI and SalI restriction sites in the pMMB67EH plasmid. The resulting complementation plasmids were transformed into respective *Ab* ATCC 17978 mutant backgrounds and induced with 2mM IPTG for complementation.

Time-dependent killing assays. Meropenem killing experiments were performed as previously described with slight alteration [125]. Target strains were grown overnight in liquid LB medium at 37°C with shaking. The following day, overnight cultures were diluted 1:10 in fresh, pre-warmed BHI^{+} medium (BD Difco Bacto Brain Heart Infusion) containing either meropenem at a final concentration of 10 mg/mL or an equivalent volume of water as blank. The diluted BHI^{+} cultures were then incubated statically at 37°C for the duration of experiment. At 0, 3, 6, 12, and 24 hours, each sample was diluted 4-fold in blank BHI^{+} medium and the optical density (OD_{600}) was measured. At each time point, cells were serially diluted 10-fold in fresh BHI^{+} broth and either 5 mL of each serial dilution was spot-plated or 100 mL of each dilution was plated on LB agar supplemented with 2% glucose for determination oof viable cell counts. Spot-plates were imaged and colonies were counted after 20 – 24 hour incubation at 37°C.

Construction of PBP7 and LdtK active site mutants. For site-directed mutagenesis, first the *pbpG* (A1S_0237) and *ldtK* (A1S_2806) coding sequences were amplified from *Ab* ATCC 17978 cDNA and cloned into the BamHI restriction site in the pUC19 plasmid and transformed into *E.*

coli C2987 chemically competent cells (New England Biolabs, Inc). The QIAprep Spin Miniprep Kit (Qiagen) was used to isolate The pUC19::*pbpG* and pUC19::*ldtK* plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen) and used as template for Pfu-mediated deletion mutagenesis (PCR components: 200 ng pUC19::*pbpG* or pUC19::*ldtK* plasmid DNA, 2 mL deletion primer #1 (5 mM stock), 2 mL deletion primer #1 (5 mM stock), 4 mL dNTP mix (New England Biolabs, Inc), 2.5 mL 10X *Pfu* reaction buffer AD (Agilent Technologies), 0.5 mL 10X *PfuTurbo* DNA polymerase AD (2.5 U/mL; Agilent Technologies) and sterile water to 25 mL total reaction volume; PCR cycling conditions: initial denaturation at 95°C for 3 minutes, total 30 cycles of 95°C for 15 seconds, 50°C for 1 minute, 68°C for 10.5 minutes and final extension at 68°C for 10 minutes). After thermocycling, 1 mL DpnI (New England Biolabs, Inc) was added directly to the PCR reactions and incubated 37°C for 1 hour. Following 1 hour of incubation, another 1 mL DpnI was added to the PCR reactions and the reactions were again incubated at 37°C for 1 hour. 5 mL of the DpnI-digested PCR reactions were then transformed into *E. coli* C2987 chemically competent cells and plated on LB agar supplemented with 75 mg/L carbenicillin. All mutants were confirmed by PCR and Sanger sequencing.

Construction of PBP7 and LdtK overexpression strains. *pbpG* (A1S_0237) and *ldtK* (A1S_2840) coding sequences were amplified from *Ab* ATCC 17978 cDNA and *pbpG*$_{S131A}$ and *ldtK*$_{C138S}$ were amplified from pUC19::*pbpG*$_{S131A}$ and pUC19::*ldtK*$_{C138S}$ plasmid DNA using primers containing 8X histidine-tag sequence (**Table SX**) and cloned into NdeI and BamHI restriction sites in the pT7-7Kn plasmid and transformed into *E. coli* C2987 chemically competent cells (New England Biolabs, Inc). Resulting pT7-7Kn::*pbpG*, pT7-7Kn::*pbpG*$_{S131A}$, pT7-7Kn::*ldtK* and pT7-7Kn::*ldtK*$_{C138S}$ were PCR confirmed and sequence verified and transformed

into *E. coli* C2527 (BL-21) chemically competent cells (New England Biolabs, Inc) for purification, expression and western blotting.

Purification of recombinant proteins. Recombinant PBP7, PBP7$_{S131A}$, LdtK, and LdtK$_{C138S}$ proteins were purified as described previously with slight modification [126]. Briefly, a single colony of *E. coli* C2527 (BL-21) cells expressing PBP7, PBP7$_{S131A}$, LdtK, and LdtK$_{C138S}$ recombinant proteins were grown overnight in LB medium supplemented with 25 mg/mL kanamycin and 2% glucose. The following day, overnight cultures were back-diluted in fresh LB medium supplemented with 25 mg/mL kanamycin and grown to OD_{600} 0.6. The cultures were then induced with 0.5 mM IPTG at 16°C with shaking for 18 – 20 hours. Following induction, the cells were harvested by centrifugation, washed with 1X phosphate buffered saline (PBS) and resuspended in lysis buffer (20mM Tris, 300mM NaCl, 10mM imidazole; pH 8.0). Sonication was carried out on ice at 80% power for 10 cycles of 20 seconds ON and 20 seconds OFF. The lysate was clarified by centrifugation to remove unlysed cells and the supernatant was incubated with 0.5 mL HisPur Cobalt resin (ThermoFisher Scientific) overnight at 4°C with rocking. The lysate/resin mixture was then applied to a flow column, washed once with lysis buffer, once with 15 mM imidazole in lysis buffer and once with 30 mM imidazole in lysis buffer. The protein was eluted in 250 mM imidazole in lysis buffer and further purified in dialysis buffer (10mM Tris, 50 mM KCl, 0.1 mm ETDA, 5% glycerol; pH 8.0) overnight at 4°C.

Western blotting. Western blot analysis was performed by transferring the protein gel to Amersham Hybond PVDF blotting membrane with a pore size of 0.45 mM (Cytive Life Sciences). Blots were then blocked with 5% milk in 1X Tris buffered saline (TBS) for 2 hours at room temperature. The 6X-His Tag Mouse anti-Tag (Invitrogen) primary antibody was diluted to 1:1000 in 5% milk and incubated with blots overnight at 4°C with gentle rocking. Blots were then

incubated with 1:5000 diluted IgG (H+L) Goat anti-Mouse HRP (Invitrogen) secondary antibody for 1 hour at room temperature. Thermo Scientific SuperSignal West Pico PLUS Chemiluminescent Substrate (ThermoFisher Scientific) was used for detection of tagged proteins.

Isolation of outer membrane vesicles. Outer membrane vesicles were isolated as described previously [127]. Briefly, overnight cultures were back-diluted to OD_{600} 0.01 in 100 mL LB and grown to stationary phase at 37°C with shaking. Cultures were then pelleted through centrifugation at 5000 x g for 15 minutes at room temperature and the supernatant was filtered through 0.45 mM bottle-top filter (Fisherbrand). Filtered supernatant was ultracentrifuged (Sorvall WX 80+ ultracentrifuge with AH-629 swing bucket rotor) at 151,243 x g for 1h at 4°C. Following final ultracentrifugation, outer membrane vesicle pellet was resuspended in 500 mL cold membrane vesicle buffer (50mM Tris, 5mM NaCl, 1mM $MgSO_4$; pH 7.5). Outer membrane vesicles were isolated three time in duplicate.

Quantification of total outer membrane vesicle proteins. Bradford assay was used to determine outer membrane vesicle protein concentration as previously described [127]. To generate a standard curve, bovine serum albumin (BSA) was diluted 0 to 20 mg/mL in Pierce Coomassie Plus assay reagent (ThermoFisher) to a final volume of 1 mL. Outer membrane vesicles were diluted 2, 5, 10, 15, 20 mL in the reagent to a final volume of 1 mL. A microplate spectrophotometer (Fisherbrand AccuSkan) was used to measure the absorbance (OD_{595}) of standard and samples in a 96-well plate (BrandTech). Protein concentrations were determined by comparing the optical densities of samples to the standard curve generated in Microsoft Excel and final quantifications were graphed in GraphPad Prism 8. Each experiment was repeated three times. One representative data set is reported.

Quantification of outer membrane vesicle 3-deoxy-D-***manno*****-oct-2-ulosonic acid (Kdo) concentrations.** Kdo assays were carried out as described previously [128]. For the standard curve, Kdo standard (Sigma) was diluted 0 to 128 mg/mL in 50 mL of DI water. 50 mL of 0.5M sulphuric acid (H_2SO_4) was added to 50 mL of isolated outer membrane vesicles and freshly prepared 50 mL dilutions of the Kdo standard. Outer membrane versicles in 0.5M H_2SO_4 were boiled for 8 mins to release the Kdo sugars. Samples were allowed to cool for 10 minutes at room temperature. 50 mL of 0.1M periodic acid was added to outer membrane vesicles and Kdo standards and incubated at room temperature for 10 minutes. Following incubation, 200 mL of 0.2M sodium arsenite in 0.5M hydrochloric acid (HCl) was added to outer membrane vesicles and Kdo standards followed by 800 mL of 0.6% freshly prepared thiobarbituric acid (TBA). All samples were boiled for 10 mins and allowed to cool at room temperate for 30-40 minutes. Prior to optical density measurements, purified Kdo was extracted using *n*-butanol equilibrated with 0.5M HCl. Optical density was measured at OD_{552} and OD_{509} (Fisherbrand AccuSkan microplate spectrophotometer) in disposable polystyrene cuvettes (Fisherbrand). A linear Kdo standard curve was generated by subtracting OD_{552} measurements from OD_{509} measurements in Microsoft Excel. Same calculations were applied to isolated OMV samples. Final Kdo concentrations were graphed in GraphPad Prism 8. Each experiment was repeated three times. One representative data set is reported.

Ethidium bromide permeability assay:

Overnight cultures were grown in 5ml BHI medium and normalize the cultures to OD_{600} to 1.5 the following day and then subculture (1:10); and with and without meropenem (10µg/ml). Cultures were withdrawn at several time points 0,6 and12 hour timepoints and washed the cells 3 times to PBS (Phosphate- buffered saline) and normalized the OD_{600} in PBS. 180 ml of the cultures was

added to 96 well black plate and 20ml EtBr were added immediately before the measurement of the fluorescence. The relative fluorescence unit was analyzed using synergy multi-mode plate reader (530 nm excitation filter, 590 nm emission filter and 570 nm dichroic mirror). The temperature was adjusted to room temperature 25°C for 30 minutes read and 15S interval. The mean RFU for each sample were calculated and plotted by Prism 9 (GraphPad 9.2.0).

Fluorescent NADA staining: Overnight cultures were grown with shaking at 37°C in 5ml of BHI liquid media. Next day, cultures were subcultures at 1:10 in fresh BHI media (total volume 5 ml) containing with and without meropenem (10 mg/ml). 2 microliters of 10mM NBD- (linezolid-7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino-D-alanine (NADA) (Thermo Fisher) was added to each tube and incubated them at 37°C stationary incubator. At different time points 6,12,24-hour, cultures (5ml) were withdrawn and washed 2 times in BHI and fixed with 1 phosphate-buffered saline containing a (1:10) solution of 16% paraformaldehyde.

Microscopy: Cells fixed with paraformaldehyde were immobilized on 1.5% agarose pads and imaged using an inverted Nikon Eclipse Ti-2 widefield epifluorescence microscope equipped with a Photometrics Prime 95B camera and a Plan Apo 100 1.45-numerical-aperture lens objective. Phase contrast and fluorescence images will be collected and analyzed using NIS Elements software. Green fluorescence images were taken using a filter cube with 632/60 or 535/ 50 emission filters and Images were captured using NIS Elements software.

Image analysis: Images were analyzed by Image Fiji. All images were minimally processed (substract background and brightness/contrast adjusted uniformly across all fluorescent images) and Pseudocolored (a Cyan lookup table was chosen to NADA images). Cells shape (length, area, width and fluorescence intensities) were quantified in MicrobeJ and data were plotted in Prism 9

(GraphPad 9.2.0). Total 300 cells were analyzed for each experiment and independently replicated three times and one representative data was reported in the quantification.

RNA sequencing. RNA sequencing was performed as described previously with modification. Briefly, the Direct-Zol RNA MiniPrep kit (Zymo Research) was used to extract total RNA from static *Ab* ATCC 17978 cultures either treated with 10 mg/mL meropenem or an equivalent volume of water as blank at 0.5, 3, and 9 hours of incubation at 37°C. RNA was then treated with Turbo DNA-free DNA removal kit (Invitrogen) to remove genomic DNA contamination. DNase-depleted RNA was sent to the Microbial Genome Sequencing Center (MiGS) for Illumina HiSeq sequencing. CLC genomic workbench software (Qiagen) was used to align the resulting sequencing data to the *Ab* ATCC 17978 genome annotations and determine the RPKM expression values and the weighted proportions fold change of expression values between meropenem treated and treated samples. The Baggerley's test on proportions was used to generate a false discovery rate adjusted P-value. The weighted proportions fold change of expression values between samples was used to generate pathway specific heatmaps in Prism 8 (GraphPad).

Minimum inhibitory concentration calculations. MICs were determined using the Broth Microdilution (BMD) method as previously outlined [129]. Overnight cultures were back diluted to OD_{600} 0.01 in Mueller-Hinton broth and 100 mL of cells was added to each well of a 96-well round-bottom polypropylene plate (Grenier Bio-One). Meropenem diluted in water was serially diluted and 150 mL of each meropenem serial dilution was also added to each well. Plates were incubated overnight at 37°C and growth was measured by reading OD_{600} after 20-24 hours of incubation. The lowest concentration of meropenem at which no bacterial growth was observed was determined to be the MIC.

Results

Meropenem susceptible Ab strains are tolerant, form spheroplasts and resume normal morphology and growth upon removal of the bactericidal antibiotic

Previous work showed that *Vibrio cholerae* [119], [120], *Pseudomonas aeruginosa* [121] and pathogens in the Enterobacterales order [130], [131] form viable, non-dividing spheroplasts when exposed to lethal concentration of beta-lactam antibiotics over several hours. Importantly, spheroplasts revert to normal rod-shaped growth when the antibiotic concentration is sufficiently reduced [130], demonstrating a short-term survival mechanism that directly contributes to antibiotic treatment failure.

To determine if populations of *Ab* strains can tolerate meropenem treatment over time, stationary phase cultures from susceptible *Ab* isolates, including recent clinical isolates, were treated with high levels (10 mg/mL; 62.5-fold MIC in strain ATCC 17978) of the antibiotic. Treated cultures demonstrated only slight depletion after 24 h, relative to untreated (**Fig 9A).** In contrast, meropenem treatment of cells in logarithmic growth phase showed rapid lysis. Therefore, *Ab* strains in stasis, a relevant physiological state during infection when the cell is known to fortify the cell envelope and slow growth/division [132], are highly tolerant to lethal meropenem concentrations. While these data agree with current dogma that beta-lactam-dependent killing is strictly proportional with growth rate [109], [133], [134], subsequent analysis revealed that stationary phase *Ab* cells experience significant cell envelope damage upon meropenem treatment. After 12 h, stationary phase cells treated with meropenem demonstrated notable morphological changes typical of spheroplast formation relative to untreated cells (**Fig 9B**). All strains showed a measurable increase in surface area and width of treated cells relative to untreated (**Fig 9C**). To visualize changes in PG assembly, cells were treated with a fluorescent derivative of D-alanine

(NADA), which is incorporated into the PG by PBPs and LD-transpeptidases [135]–[138]. A significant decrease in NADA intensity was evident in meropenem treated cultures relative to untreated at 12 h **(Fig 9B &9D),** suggesting degradation of the cell wall, as previously shown in other beta-lactam tolerant Gram-negative bacteria [119], [130]. Thus, tolerance under stationary phase conditions is not just a simple function of growth inhibition, but rather an active response to significant cell envelope damage.

Since *Ab* spheroplasts were viable after plating **(Fig 9A),** we also wanted to determine if the characteristic *Ab* coccobacilli morphology was restored after antibiotic removal. Cells treated with meropenem for 12 h were washed and grown in fresh media without antibiotic. At 12 h post-treatment, no spheroplast were found after antibiotic removal **(Fig 9E),** wild type morphology was restored **(Fig 9F)** and the cells showed incorporation of NADA suggesting PG was being synthesized and remodeled **(Fig 9E).** Fluorescence intensity measurements showed a stepwise decrease in fluorescence intensity at 6 and 12 h after treatment started relative to the start of the experiment (0 h) (Fig 9G), suggesting PG degradation during meropenem treatment. After 12 h of meropenem treatment, cells were washed to remove the antibiotic, resuspended in fresh media, and stained with NADA. Fluorescence intensity measurements 12 hours post meropenem treatment showed increased NADA incorporation **(Fig 9G),** suggesting the cell resumed PG remodeling and synthesis. Furthermore, fluorescence intensity appeared higher at the midcell of some cells **(Fig 9E, white arrows).** Divisome components localize to the midcell where they regulate daughter cell formation, suggesting the recovered population had resumed division. Together, these data support a model where *Ab* spheroplasts revert to wild type morphology and growth when meropenem treatment is stopped.

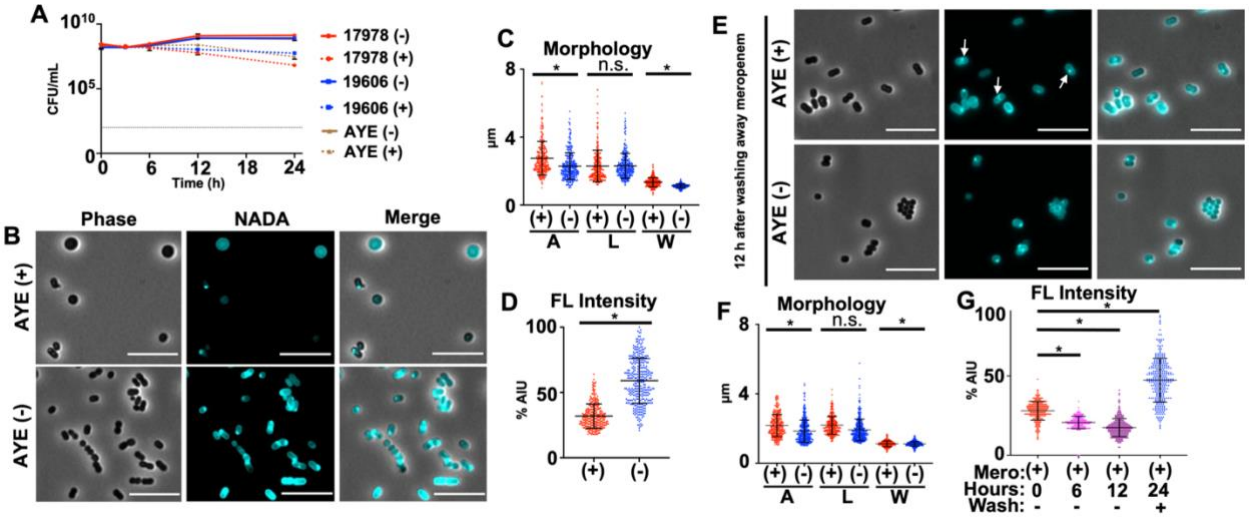

Figure 9: *Ab* strains are tolerant to meropenem. (A) Colony-forming units (CFUs) of *Ab* strains ATCC 17978, 19606 and AYE untreated (-) or treated (+) with meropenem over 24 h. Each killing assay was independently replicated three times, and one representative dataset was reported. Error bars indicate SD. Dotted black line indicates level of detection. (B) Phase and fluorescence microscopy of + or - *Ab* strain AYE after 12 h. Scale bar is 10 µm. (C) Area (A), length (L) and width (W) quantitation of cells in panel B (*n*= 300). (D) Fluorescence (FL) signal intensity quantitation in percent arbitrary intensity units (AIU) of treated vs. untreated cells in panel B (*n*= 300). (E) After 12 hours of meropenem treatment, the cells were washed with fresh media without antibiotic and stained with NADA. Cells were imaged after 12 h and showed the characteristic *Ab* coccobacilli morphology is restored. White arrows indicate fluorescence intensity at the midcell. (F) Area (A), length (L) and width (W) of cells in panel E (*n*= 300). (G) FL signal intensity in percent AIU treated vs. untreated (*n*= 300) at 0, 6 and 12 hours after meropenem treatment and 12 h after removal of the antibiotic. Significance was determined using an unpaired t-test ($P < 0.05$) in treated vs. untreated. An asterisk indicates significant differences between treated and untreated; n.s., not significant. Error bars indicate SD from the mean.

Transcriptome analysis highlights differentially regulated pathways important for Ab tolerance

Many Gram-negative pathogens rapidly form spheroplasts to develop meropenem tolerance [119], [130]; however, *Ab* spheroplast formation is delayed. We first observed spheroplast formation only after 8 h, with large numbers within the population accumulating by 12 h **(Fig 9 B & C)**. To define transcriptional alterations associated with spheroplast-associated tolerance, we isolated RNA from treated and untreated cells at 0.5, 3 and 9 h. While subtle changes in gene expression were evident at 0.5 and 3 h, differential expression patterns were more obvious at 9 h in treated cultures relative to untreated **(Fig 10)**. Genes associated with efflux were increasingly upregulated with each timepoint (Fig 10 A), suggesting the cell quickly and continually responds to meropenem treatment by actively expelling the toxic compound. Upregulated efflux genes included *adeAB, adeIJK* and *macABtolC,* which have all been implicated in antibiotic efflux [139]–[141]; specifically, beta-lactam efflux is associated with the AdeIJK RND-type pump [142], [143]. To validate the role of AdeIJK in meropenem tolerance, we constructed a genetic knockout (D*adeIJK*), which was subjected to high-level meropenem treatment **(Fig 10 B)**. At 24 h post-treatment, D*adeIJK* was more than 1,000-fold more susceptible to meropenem-mediated killing relative to wild type, showing that efflux contributes to tolerance.

Porins represent the major entryway for carbapenems such as meropenem to enter the periplasm [144], where they inhibit transpeptidation to cross-link the stem peptides of adjacent PG strands. Decreased expression of many porin-associated genes was evident in treated cultures relative to untreated **(Fig 10 C),** suggesting the cell also limits meropenem entry by reducing porin gene expression in response to treatment. However, temporal expression of porin-associated genes was delayed relative to efflux, in general. Deletion of *carO* is associated with carbapenem resistance in *Ab* [145] and was found to be an influx channel for carbapenems [146], while OprD

has also been associated with clinical carbapenem resistance in *Ab* [147], suggesting reduced expression may strategically limit meropenem entry. Interestingly, the largest reduction in gene expression was associated with *ompW*, which encodes a predicted b-barrel protein (OmpW) that supports iron uptake [148], but our understanding of its biological function or how it contributes to carbapenem resistance or tolerance is limited. Notably, in *Vibrio cholerae*, decreased iron uptake regulated by the VxrAB two-component system, promotes spheroplast recovery by reducing oxidative stress during beta-lactam treatment [149], [150]. To further validate the RNA-seq dataset, we analyzed D*ompW* in a meropenem killing assay, which showed approximately a 10-fold increase in survival relative to wild type (**Fig 10B**).

Consistent with published *Ab* transcriptional datasets in stress [151]–[153], meropenem treatment also induces expression of genes encoding putative outer membrane lipoproteins and their transporters (LolA-D) (**Fig 10D**). Outer membrane lipoproteins fortify the *E. coli* cell envelope by providing structural rigidity, where inner leaflet outer membrane lipoproteins are covalently attached to the underlying PG network [103], [154]. Lastly, we made an isogenic mutant of *A1S_3492*. D*A1S_3492* showed approximately a 100-fold increase in meropenem susceptibility relative to wild type **(Fig 10B).** Together, analysis of efflux, porin and lipoprotein mutants suggest a transcriptional response that protects *Ab* from meropenem-dependent killing during treatment.

Transcription of genes associated with PG remodeling were only slightly altered with the notable exception of two genes encoding putative LTs , including membrane bound, MltF, and a soluble protein, Slt, which were both upregulated (**Fig 10 E**). LTs cleave *N*-acetylmuramic acid (Mur*N*Ac)-*N*-acetylglucosamine (Glc*N*Ac) bonds in PG to release soluble 1,6-anhydroMur*N*Ac-containing muropeptides. Muropeptides excised by LTs can be secreted into the environment or imported into the cytoplasm and catabolized via the PG recycling pathway [155]. 1,6-

AnhydroMur*N*Ac-containing muropeptides that feed into PG recycling can act as a source of nutrients, but also can be re-incorporated into the PG network through *de novo* biosynthesis or in some bacteria can also act as signals to induce beta-lactamase expression [156], [157]. Lastly, genes involved in lipooligosaccharide (LOS), and PG biosynthesis were slightly altered (**Fig 10 FG).**

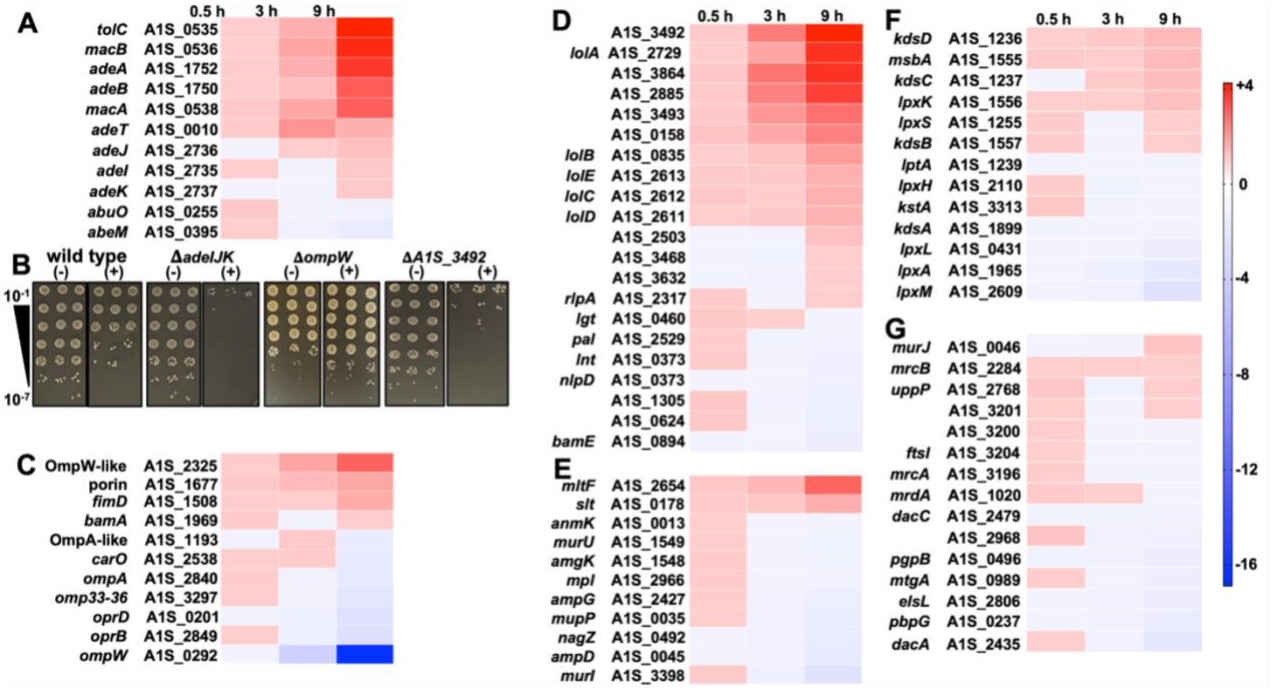

Figure 10: Differentially regulated genes in response to meropenem treatment in *Ab*. Heat map showing the fold-change in genes expressed at 0.5, 3 and 9 h meropenem treatment relative to wild type ATCC 17978 (P <0.05). (A) Differentially regulated genes associated with efflux. (B) Dilution spot assays (in triplicate) of wild type, **Δ***adeIJK*, **Δ***ompW* and **Δ***A1S_3492* with (+) or without (-) meropenem treatment. (C) Same as (A), but pathway analysis includes genes associated with outer membrane porins, (D) outer membrane lipoproteins and their transporters, (E) PG recycling, (F) LPS biosynthesis and (G) PG biosynthesis.

Genes and pathways that contribute to Ab fitness during meropenem treatment

While transcriptome sequencing analyses offer insight into the stress response, one limitation of RNA-sequencing is that differentially regulated genes oftentimes do not impact fitness due to redundancy or pleiotropic effects. Therefore, we also performed transposon-sequencing on *Ab* strain ATCC 17978. Using previously constructed high-density transposon libraries generated in wild type *Ab* [158], [159], stationary phase cultures were either treated with meropenem or left untreated during incubation at 37 degrees. After six, nine and 12 h, cells were collected, insertions were mapped and comparisons between treated and untreated cultures were used to identify meropenem tolerance factors. The screen was answered by several novel factors, some of which are the subject of a separate study, but also revealed the importance for outer membrane integrity and PG maintenance (**Fig 11A).** To validate our screen, we calculated survival in several mutants, including *⍙ompA, ⍙lpxM, ⍙pbpG* and ⍙elsL (also known as *ldtK* [160]), in the presence and absence of meropenem (Fig 11B). All mutants showed at least a 2-to-3-fold log depletion relative to wild type at 12 h and >5-fold log depletion at 24 h. Importantly, the meropenem minimal inhibitory concentrations did not change significantly in the mutants relative to wild type (**Fig 11C)**. These studies suggest that *Ab* fitness during meropenem treatment is dependent on outer membrane integrity and PG maintenance factors.

OmpA is a highly conserved monomeric b-barrel protein with a periplasmic domain that noncovalently attaches the outer membrane to the PG network [161]. It is highly abundant in *Ab* [162] and coordinates with efflux pumps to export antibacterial compounds from the periplasm [163], [164]. OmpA is also known to stabilize the outer membrane; *ompA* deletion/disruption induces outer membrane vesicle formation and increases permeability [165]. To test the hypothesis that *ompA* deletion perturbs the outer membrane to promote meropenem entry in *Ab*, we performed

two assays, including permeability measurements (**Fig 11D**) and quantified outer membrane vesiculation (Fig 11E). Relative to wild type [165], △*ompA* induced outer membrane vesicle formation and permeability to ethidium bromide, which is similar in size to meropenem. We also measured ethidium bromide influx in △*lpxM, ⊿pbpG and* △*elsL* (**Fig 11D**). Like △*ompA,* all isogenic mutations increased permeability relative to wild type and the respective complementation strain, which restored the permeability defect. Notably, meropenem treatment did not exacerbate permeability in wild type or any of the mutants **(Fig 11D),** suggesting it does not directly destabilize the outer membrane barrier function. Since we previously reported that △*elsL* produces excess outer membrane vesicles [158] and all of the mutants showed increased permeability, we also tested vesicle formation in △*lpxM* and △*pbpG* (**Fig 11E).** Unexpectedly, △*pbpG* produced excess outer membrane vesicles relative to wild type and all other mutants. In contrast, *⊿lpxM* did not.

Interestingly, △*lpxM* was the only strain that showed increased permeability but not hypervesiculation. LpxM catalyzes transfer of two lauroyl ($C_{12:0}$) groups from an acyl carrier protein to the *R*-3*'*- and *R*-2-hydroxymyristate positions of lipid A during LOS biosynthesis [166]. Mutations that reduce LOS acylation are known to increase fluidity of the lipid bilayer and could also impact folding/function of outer membrane porins [167], [168]. Either/both mechanisms could increase entry of meropenem into the periplasmic space or disrupt efflux mechanisms that actively pump the compound out of the cell.

We also characterized the morphology of each mutant in growth. We found that relative to wild type, △*ompA* cells were chained and NADA incorporation was reduced, suggesting that OmpA is required for proper function of PG enzymes (division proteins and LD-/DD-transpeptidases that incorporate NADA and/or increased carboxypeptidase activity). △*lpxM* and

⊿pbpG showed increased NADA incorporation which is consistent with increased outer membrane permeability. *⊿pbpG* cells were also clumped, suggesting the cells could not properly separate during division. As previously reported [158], *⊿elsL* showed rounded cells.

While the role of OmpA and LpxM to gate meropenem entry into the *Ab* periplasm to promote antibiotic tolerance is straightforward, we were intrigued by genetic links to PG maintenance (i.e., *pbpG* and *elsL*). While mutation of *pbpG* and *elsL* impact outer membrane integrity (**Fig 11**), we also wanted to define their physiological role to determine specific pathways that contribute to meropenem tolerance.

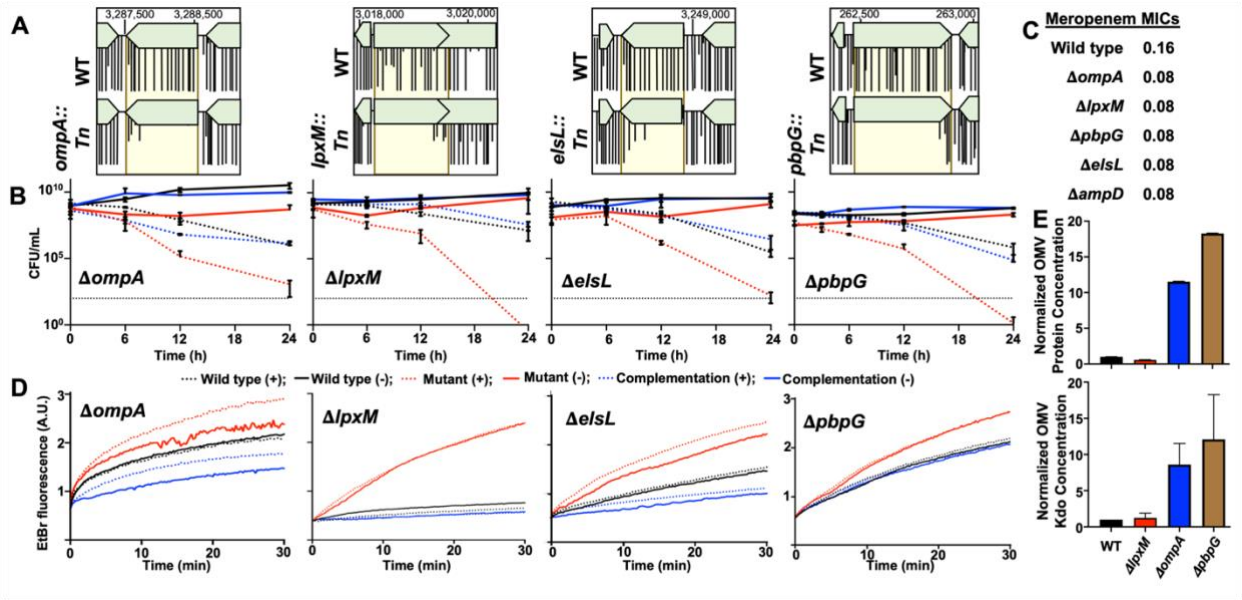

Fig 11: Genes encoding outer membrane integrity and PG maintenance contribute to meropenem tolerance in *Ab*. (A) Tn-seq analysis of genes required for meropenem tolerance at 12 h. (B) Survival in isogenic mutants was calculated as CFU/mL over 24 h during meropenem treatment. Data were collected from two experiments in triplicate. Error bars represent the average of 3 technical replicates +/- SD (C) Minimal Inhibitory Concentrations (MICs) of wild type and

isogenic *Ab* mutants. (D) Permeability assays using ethidium bromide (EtBr) over 0.5 h. A.U.; arbitrary units. Lines depict the mean of three technical replicates. (E) Relative quantification of protein (top) and Kdo (bottom) concentrations of outer membrane vesicles (OMVs) in wild type (WT) and mutants. Each experiment was independently replicated three times, and one representative dataset was reported. Error bars indicate SD. An asterisk indicates significant differences relative to the WT strain (P <0.05).

PBP7 is a DD-carboxypeptidase and endopeptidase that catalyzes formation of tetrapeptides

To define the activity of *Ab* PBP7 (encoded by *pbpG*), we isolated PG from wild type and △*pbpG* in growth (**Fig 12A)** and stasis (**Fig 12B)** Muropeptides were generated by treatment with muramidase, separated by high-performance liquid chromatography and uncharacterized peaks were analyzed by tandem mass spectrometry as done previously [151], [158], [169]. PG composition from △*pbpG* in growth showed accumulation of two muropeptide peaks that were not present in wild type (**Fig 12A**). MS analysis showed these peaks were enriched with pentapeptides and were identified as disaccharide pentapeptide (Penta, neutral mass: 1012.19 amu; theoretical: 1012.45 amu) and bis-disaccharide tetrapentapeptide (TetraPenta, neutral mass: 1935.60 amu; theoretical: 1935.84 amu) (**Fig 12**), suggesting the enriched muropeptide pools represent PBP7 substrates in growth. △*pbpG* PG in stasis had depleted D-amino acid-modified muropeptide pools, including TetraTri-D-Lys- and TetraTri-D-Arg-peptides, and reduced 3-3 crosslink formation (**Fig 12BC**) consistent with PBP7 DD-carboxypeptidase and endopeptidase activity to form tetrapeptides, which are the most abundant peptides in the PG in *Ab* [151], [158], [169] and substrates of LD-transpeptidases. The periplasmic LD-transpeptidase, LdtJ, transfers D-amino acid to tetrapeptides and forms 3-3 crosslinks [158]. Therefore, it is likely that PBP7

provides at least some of the periplasmic substrates for LdtJ-dependent transpeptidase activity in stasis.

To test the enzymatic activity, we purified recombinant PBP7)and a predicted catalytically inactive version in which alanine replaces the active site serine ($PBP7_{S131A}$). Purified proteins were incubated with PG from *E. coli* D456 (**Fig 12 D),** a strain enriched with pentapeptides [170] and analyzed as previously done [171]. PBP7 was active against penta-, tetratetra- and tetrapentapeptides, where each muropeptide was trimmed to the tetrapeptide-form relative to the no-enzyme control. As expected, $PBP7_{S131A}$ did not show activity against any muropeptides. Together, these studies suggest that PBP7 not only hydrolyzes the bond between the terminal D-Ala residues, but also showed DD-endopeptidase activity, where both activities enrich the periplasmic pool of monomeric tetrapeptides.

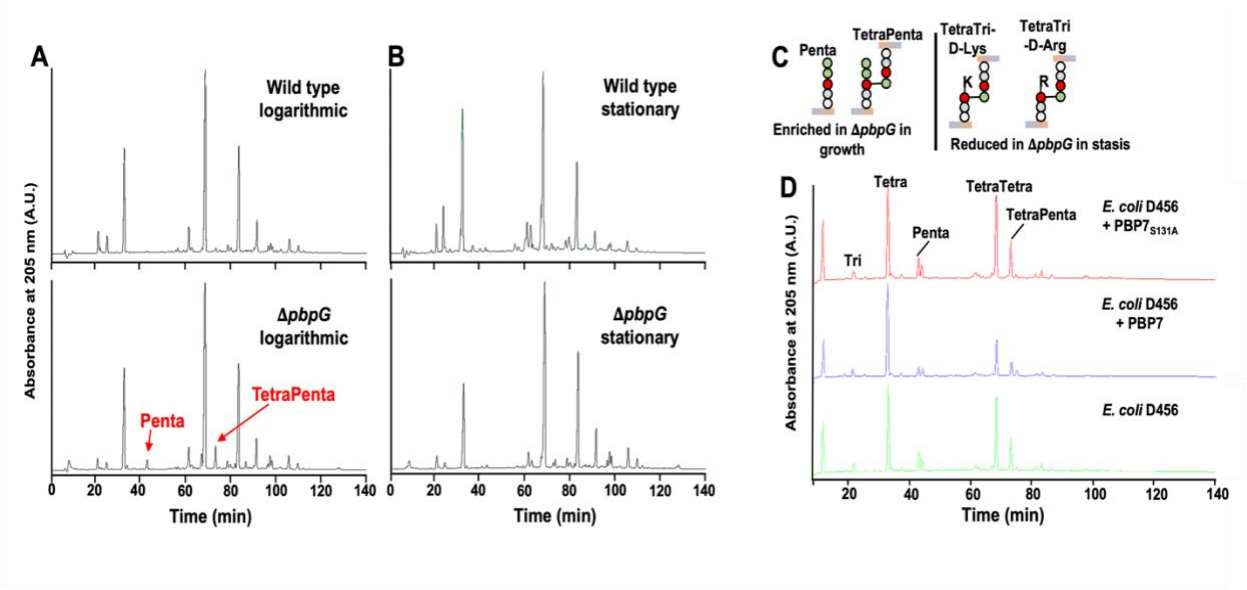

Figure 12: PBP7 is active against pentapeptides and DD-crosslinks. (A) PG isolated from wild type and ∆*pbpG* in growth phase was analyzed by HPLC. The muropeptides Penta and TetraPenta

were enriched in △*pbpG*. (B) PG isolated from wild type and △*pbpG* in stationary phase was analyzed by HPLC. TetraTri-D-Lys and TetraTri-D-Arg were depleted in △*pbpG* relative to wild type. (C) Muropeptide structures are illustrated and were confirmed using MS/MS. (D) Recombinant PBP7 or the active-site mutant $PBP7_{S131A}$ was incubated with PG isolated from *E. coli* D456 which contains Tetra, Penta, TetraTetra and TetraPenta as the main muropeptides. PBP7 was active against pentapeptides (DD-CPase) and cross-linked muropeptides (DD-EPase).

ElsL is a cytoplasmic LD-carboxypeptidase active against tetrapeptides for PG recycling

During beta-lactam treatment, autolysins (i.e., LTs) are activated [109], [172], which increases the amount of PG turnover products with 1,6-anhydro-Mur*N*Ac residues. In *Ab*, genes encoding the autolysins, MltF and Slt, were upregulated during meropenem treatment (**Fig 10E**), which likely increases periplasmic concentrations of TetraAnh, for cytoplasmic import. In *E. coli*, TetraAnh are substrates for the LD-carboxypeptidase LdcA, which trims tetrapeptides to tripeptides [173] that are catabolized by the conserved enzymes NagZ [174], [175] to generate 1,6-anhMur*N*Ac-tripeptide and the amidase AmpD [176], [177] to form free tripeptides, which can be further broken down into individual amino acids and used as energy. Furthermore, Mpl [178] can attach tripeptides to uridine diphosphate (UDP)-Mur*N*Ac to form UDP-Mur*N*Ac-tripeptide, an intermediate in the *de novo* PG biosynthesis pathway. However, no apparent LD-carboxypeptidase, orthologue to LdcA is encoded by *Ab*.

ElsL was one potential LD-carboxypeptidase candidate for PG recycling because it encodes a putative LD-transpeptidase (YkuD) domain but does not encode a canonical secretion signal needed for export, suggesting it may be active in the cytoplasm. This observation coupled with a recent study showing that the *E. coli* YkuD homologue DpaA (also known as LdtF) is an

amidase that hydrolyzes bonds formed by LD-transpeptidases [104], [179], suggested that ElsL may indeed have LD-carboxypeptidase activity, which is essential for tripeptide formation in the recycling pathway.

First, we determined the subcellular localization of ElsL with a specific antibody that detects the native protein (**Fig 13A).** After fractionation of the subcellular compartments, we were only able to detect ElsL in the cytoplasmic fraction in growth and stasis, showing it is not exported to the periplasm.

Since ElsL is cytoplasmic, we sought to determine if it was active on tetra- and/or pentapeptide substrates. We purified recombinant ElsL and the active-site mutant, $ElsL_{C138S}$. Both enzymes were incubated with muropeptides obtained from tetrapeptide-rich PG from *E. coli* BW25113 or pentapeptide-rich PG from *E. coli* CS703-1 [171] (**Fig 13B**). ElsL showed activity against tetrapeptides but not pentapeptides. The muropeptide profile showed the formation of disaccharide tripeptide and bis-disaccharide tetratripeptide, showing that ElsL cleaves the bond between the L-centre of *m*DAP and the terminal D-Ala in tetrapeptides, characteristic of LD-carboxypeptidase activity. Our data indicate that PBP7 trims pentapeptides and cleaves crosslinked peptides into tetrapeptides in the periplasmic PG network. Once 1,6-anhydro-Mur*N*Ac-containing muropeptides are released from the PG network by MltF, Slt or other LTs , they are transported into the cytoplasm and processed into tripeptides by ElsL in the PG recycling pathway.

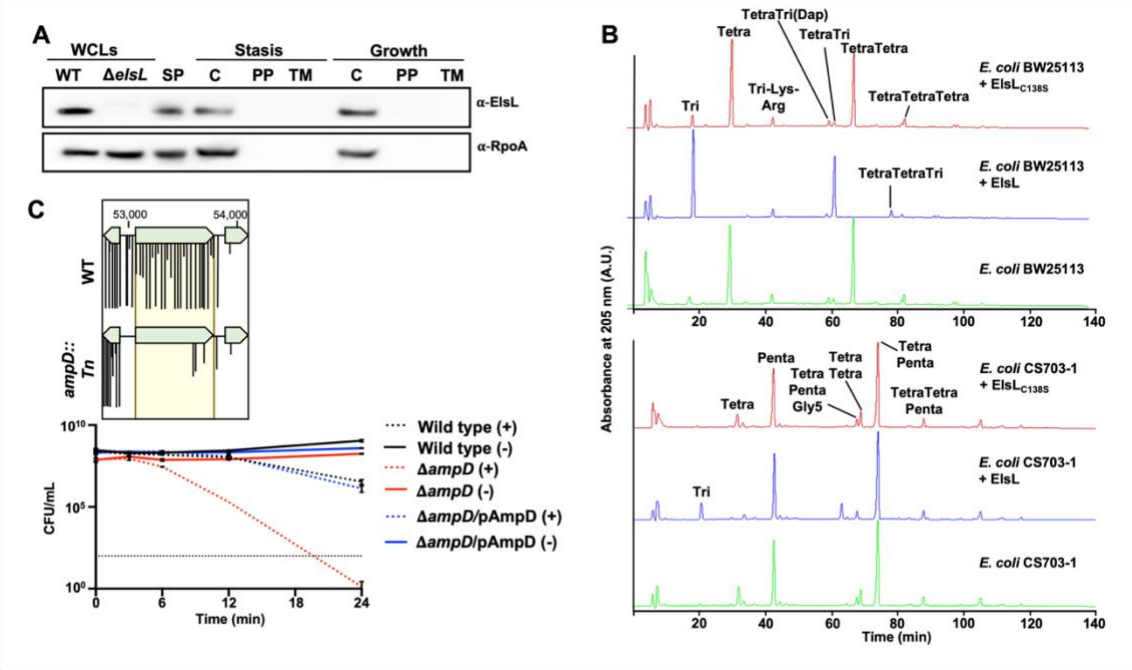

Figure 13: ElsL is active against tetrapeptides in Tetra and TetraTetra. (A) Western blot with **α**-ElsL and **α**-RpoA antisera. ElsL is 18.97 kDa, while RpoA is 37.27 kDa. WCL; whole-cell lysate, SP; spheroplast, C; cytoplasm, PP; periplasm, and TM; total membrane fractions. (B) Recombinant ElsL or the active-site mutant $ElsL_{C13S}$ was incubated with PG isolated from *E. coli strain* BW25113 (top, tetrapeptide-rich) or strain CS703-1 (bottom, pentapeptide-rich). ElsL was active against tetrapeptides but not pentapeptides. (C) Tn-seq analysis of *ampD::Tn* insertions relative to wild type 12 h after meropenem treatment. (D) Meropenem tolerance assay in △*ampD*, which encodes a well-conserved cytoplasmic enzyme required for PG recycling. Error bars represent the average of 3 technical replicates +/- SD.

To further confirm if the cytoplasmic PG pathway contributes to meropenem tolerance in *Ab*, we made an isogenic *ampD* mutant, which encodes *N*-acetylmuramyl-L-alanine amidase that

releases the tripeptide from anhMur*N*Ac [177]. Importantly, *ampD* transposon insertions were also depleted in the meropenem tolerance screen (**Fig 13C**). Like △*pbpG* and △*elsL*, △*ampD* was also rapidly killed when treated with meropenem relative to wild type and the respective complementation strain **(Fig 13D)**. Together, these studies strongly suggest that the PG recycling pathway contributes to meropenem tolerance in *Ab*. Furthermore, formation of cytoplasmic tripeptides or tetrapeptides appears to contribute to meropenem tolerance. Combinatorial therapies that inhibit enzymes in both PG biosynthesis and recycling could provide an alternative treatment strategy.

Discussion

Many susceptible Gram-negative pathogens tolerate treatment with bactericidal antibiotics such as carbapenem beta-lactams, but the molecular factors that underlie cell survival are not understood. Significant cell envelope damage is observed during treatment in stasis, characterized morphologically by cell wall-depleted spheroplasts [119]–[121], [130], [131]. Here, we show meropenem treatment induces spheroplast formation in *Ab*, and that cell growth resumes upon removal of the antibiotic. Transcriptome sequencing analysis suggested *Ab* responds to meropenem treatment by fortifying the structural integrity of the cell envelope through increased outer membrane lipoprotein and transporter gene expression and by inducing autolysins, which likely physically reinforce the envelope by providing stiffness and remodeling the PG network, respectively (**Fig 14**). Meropenem treated cells also appear to limit periplasmic concentrations through induced expression of efflux-associated genes and downregulation of porin genes, which both reduce periplasmic concentrations by actively pumping the antibiotic out of the cell and by limiting entry, respectively. A separate genetic (transposon) screen to identify fitness determinants,

showed factors required for high level meropenem tolerance include genes that contribute to outer membrane permeability (*lpxM*, *ompA*, *pbpG* and *elsL*) and cell envelope stability (*ompA*, *pbpG* and *elsL*). Furthermore, genes in the cytoplasmic PG recycling pathway, *elsL* and *ampD*, also answered the screen. Together the transcriptomics and genetic screen suggested that factors working to maintain cell envelope homeostasis through integrity maintenance of the outer membrane and PG network, contribute to meropenem tolerance in *Ab* (**Fig 14).**

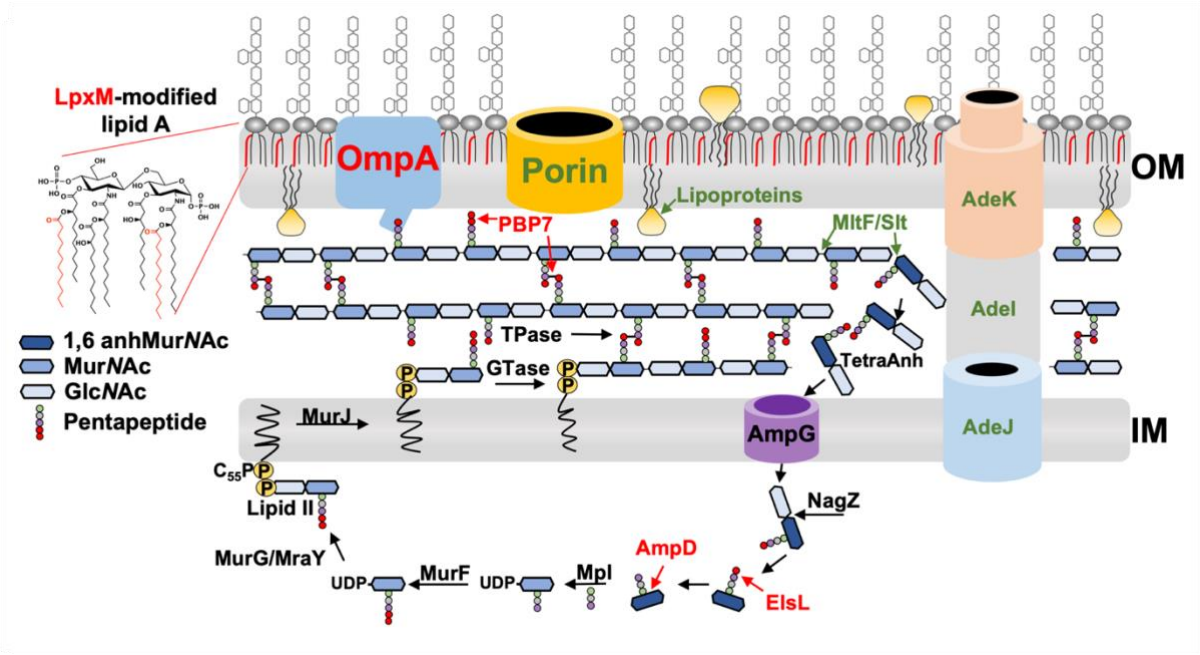

Figure 14: Model showing mechanisms that promote meropenem tolerance in *Ab.* Based on transcriptomic analysis, several genes are differentially expressed after treatment with high levels of meropenem. Pathways include upregulation of the AdeIJK efflux pump, lipoproteins, the MltF/Slt autolysins, while porins, including CarO and OprD, were downregulated (green). Fitness screens showed that several genes involved in outer membrane, periplasmic and cytoplasmic pathways promote meropenem tolerance. Pathways include OmpA, which tethers the outer membrane to the PG, LpxM, which increases outer membrane hydrophobic packing, the DD-

carboxypeptidase and endopeptidase, PBP7, and cytoplasmic recycling enzymes, ElsL and AmpD (red).

While we showed several tolerance factors are transcriptionally regulated, we do not know what transcriptional regulators are involved. A previous study found that PhoPQ-dependent outer membrane modifications promoted survival in cell wall-deficient spheroplasts [131], presumably by fortifying the outer membrane to counter large loads of turgor pressure typically absorbed by the cell wall. Specifically, PhoPQ was activated in response to meropenem treatment. *Ab* does not encode PhoPQ, but analogous mechanisms are likely to contribute to cell envelope homeostasis to counter the turgor when the cell wall is compromised. One mechanism might include fortification of the cell envelope with lipoproteins, which occurs in *Ab* during stress [151]–[153], [180]; however, the underlying protective mechanism is not understood. Landmark studies in cell envelope mechanics have shown that outer membrane lipoprotein attachment to the PG impact cell envelope mechanics by increasing the load-bearing capacity of the cell envelope [103], [154]. Outer membrane lipoproteins, specifically those that interact with the underlying PG network, increase outer membrane stiffness, which likely counterbalances the internal turgor. When the cell wall is perturbed during meropenem treatment, small fluctuations in turgor may be sufficient to induce lysis when lipoprotein-mediated attachment is absent. More studies are needed to tease apart the contribution of specific lipoproteins and how they contribute to cell envelope mechanics in stress. Furthermore, noncovalent attachments between the outer membrane and PG network via OmpA and hyperacylation of lipid A via LpxM may also increase the mechanical load-bearing capacity of the outer membrane to maintain envelope homeostasis when the cell wall is defective. It is also possible that disruption of OmpA or LpxM induced pleiotropic effects that reduced the barrier function to gate meropenem entry.

Unexpectedly, our data suggest that PG maintenance enzymes contribute to *Ab* survival during meropenem treatment. Tetrapeptides represent the most abundant PG stem peptides in *Ab.* They are formed, in part, by the DD-carboxypeptidase and endopeptidase activity of PBP7 on pentapeptides and DD-crosslinked muropeptides, respectively (**Fig 12A**). Tetrapeptides are substrates for LD-transpeptidase that form a small amount of 3-3 crosslinks in *Ab*, but are needed to effectively repair PG defects in stressed *E. coli* cells [104]. Furthermore, tetrapeptides are also necessary for LD-transpeptidase-dependent covalent attachment of Braun's lipoprotein (Lpp) to *meso*-DAP residues in PG [181], which also fortifies the envelope [103]. Our data indicate that without PBP7, LdtJ-dependent 3-3 crosslink formation is reduced (**Fig 12A**), however less than 3% of all muropeptides contain 3-3 crosslinks and their contribution to PG integrity maintenance remain unclear.

The LTs MltF and Slt were induced in meropenem treatment **(Fig 10 & 14)**, consistent with activation of autolysins in response to penicillin-binding protein inhibition during beta-lactam treatment [109], [119]. LT proteins cleave the glycosidic linkage between disaccharide subunits within the PG strands and perform an intramolecular transglycosylation in Mur*N*Ac to release soluble 1,6-anhydroMur*N*Ac-containing muropeptides, which can be imported into the cytoplasm. The main turnover product, TetraAnh is transported into the cytoplasm by AmpG where they provide substrates for ElsL-dependent LD-carboxypeptidase activity to form TriAnh. Like other members of the YkuD family, ElsL retains preference for tetrapeptide substrates (**Fig 13B**) but represents the first known YkuD-containing enzyme that lacks a signal sequence and is active in the cytoplasm, consistent with recent findings from other groups [182], [183]. ElsL is the second member of the YkuD family, after DpaA/LdtF that has a major role in cleaving amide bonds rather than generating them. Notably, the requirement for ElsL in meropenem tolerance is like how cells

depend on outer membrane integrity maintenance (OmpA, LpxM, PBP7) during spheroplast formation. Lastly, ElsL (and LdtJ) were shown to be essential for *Ab* survival without LOS [158], suggesting that PG recycling and modification of tetrapeptides are a general response to counter cell envelope stress in *Ab*. It is also possible that cytosolic accumulation of tetrapeptides creates another problem in cells that are already sick and cannot be tolerated.

Hydrolysis of TriAnh by the dedicated enzymes NagZ and AmpD, which lead to the formation of anhMur*N*Ac-tripeptide, 1,6-anhMur*N*Ac and tripeptides, respectively, could be degraded into individual amino acids for utilization as nutrient or energy sources [156], [184], [185] to promote survival during tolerance. It is reasonable to expect the cell requires some nutrients during tolerance, and this pathway could provide energy to support basal metabolic processes. Alternatively, Mpl could ligate tripeptides to UDP-Mur*N*Ac in the recycling pathway [178], [186]. UPD-Mur*N*Ac-tripeptide is an intermediate in the *de novo* PG synthesis pathway [187]–[189]; however, it is not obvious how *de novo* PG synthesis via recycling would benefit the bacterium during treatment because periplasmic PBPs and LD-transpeptidases, which are required for crosslinking, are both inhibited by meropenem. Another possibility is that accumulation of cytoplasmic 1,6-anhydroMur*N*Ac-containing muropeptides provide signals to induce beta-lactamase expression, which could localize in the periplasm to reduce meropenem concentrations to survivable levels. Two mechanisms have been characterized in Gram-negative bacteria, including the AmpG-AmpR pathway and the BlrAB two-component system, which both induce beta-lactamase expression in response to muropeptide concentrations [157]. Many genes in *Ab* have not yet been characterized. Signaling pathways and potentially carbapenemases could be induced in response to 1,6-anhydroMur*N*Ac-containing muropeptide accumulation to promote meropenem degradation. A more detailed analysis is needed to characterize the PG recycling

tolerance mechanism, which will inform more effective treatment strategies to combat *Ab* infections. Furthermore, our studies also show that disruption of PG maintenance enzymes (i.e., PBP7, ElsL) compromised outer membrane integrity. It is also possible that outer membrane perturbations in these mutants induce unchecked antibiotic entry to impact fitness during meropenem treatment. Notably, regulatory links between the outer membrane and PG maintenance are not well-understood in *Ab*.

CHAPTER 4

PEPTIDOGLYCAN RECYCLING PROMOTES ELONGATION AND OUTER MEMBRANE HOMEOSTASIS IN *ACINETOBACTER BAUMANNII.*

Nowrosh Islam[1] Samantha Quintanilla[2], Tahj Starr[3], Enrique Rojas[3], Waldemar Vollmer[4], and Joseph Boll[2]

[1]Department of Biology, University of Texas at Arlington, Arlington, Texas, USA

[2]Department of Microbiology, University of Dallas, Texas, USA

[3]Department of Biology, Newyork University, Newyork, USA

[4]Centre for Bacterial Cell Biology, Biosciences Institute, Newcastle University, Newcastle upon Tyne, United Kingdom

**In preparation:

Abstract:

The bacterial cell wall is a dynamic matrix constantly subjected to remodel for the growth and division. Cell walls remodeling involved the cleavage of preexisting cell wall material by the activity of several autolysins and subsequent insertion of new cell wall material by PG synthases. The cell wall fragments cleaved by autolysin can be recycled and act as a messenger of diverse cell signaling events and contributes to antibiotic resistance. Many Gram-negative pathogens use cell wall recycling pathway as a defensive mechanism of activating beta-lactamases through the AmpC pathway. The hospital-acquired Gram-negative nosocomial pathogen *Ab* shows resistance to all the conventional drugs including beta-lactam. Along with resistance, our previous study showed that susceptible clinical isolates of *Ab* show high tolerance to beta-lactam antibiotic meropenem, and cell wall recycling promotes outer membrane integrity and enables bacteria to survive longer. In this current study, we determined beta-lactamase expression is not important during muropeptide recycling in the presence of meropenem. Interestingly, we found that mutation in a single recycling gene impacts the rod/elongation activity in *Ab*. Here, we demonstrated that PG recycling promotes cell elongation. Moreover, we showed overexpression of ElsL and AmpD slightly impact cell elongation. Overall, this study emphasizes cell wall recycling is critical for bacterial growth and maintaining cell membrane homeostasis in *Ab*.

Introduction:

PG is a complex polymer that helps maintain the structural integrity and the shape of the bacterial cell[190] [190]–[192]. Along with PG, outer membrane (OM) has also a role in determining bacterial shape[191]. Our previous characterization showed that both outer membrane proteins and PG recycling gene products coordinate to promote bacterial survival

during meropenem treatment. We also found that disruption of PG recycling genes affects meropenem susceptibility. A recent study showed that PG recycling genes are important for bacterial fitness and virulence in *Ab* [193]. However, how this PG recycling pathway works is yet to be known. PG recycling genes are used by *Pseudomonas aeruginosa* and several Gram-negative pathogens to induce beta-lactamase that hydrolyzes or degrade the activity of the beta-lactam antibiotic and cause antibiotic resistance [194],[195].

In this study we explored if *Ab* also induces beta-lactamases since *Ab* universally encoded two beta-lactamases on its chromosome, bla_{ADC} (AmpC) and $bla_{OXA\text{-}51}$ [196]. Here we showed that removal of either beta-lactamase does not impact bacterial fitness during meropenem treatment. We also highlighted that deletion of PG recycling genes induced phenotypic defects that were identical to the rod system mutants. Also, antibiotic susceptibility was altered accordingly. Additionally, we found that overexpression of AmpD (1,6-anhydro-N-acetylmuramyl-L-alanine amidase) or ElsL (LD-carboxypeptidase) to wild type were not sufficient to induce dramatically elongated cells, but cell width indicated increased rod activity. These studies suggest that PG recycling may coordinate with the rod system to induce axial PG synthesis.

Materials and Methods:

Bacterial strains and growth: All strains and plasmids used in this study were initially grown from freezer stocks on solid agar at 37° C. Isolated colonies were used to inoculate Luria-Bertani (LB) or Brain heart infusion (BHI*)* medium. Antibiotics were used at the following concentrations unless noted otherwise: 25 mg/L kanamycin, 10 mg/L meropenem, 10 mg/L tetracycline and 75 mg/L carbenicillin.

Fluorescent NADA staining: Overnight cultures were back diluted to OD_{600} of 0.05 and grown at 37° C in LB media until they reached stationary or mid-logarithmic growth phase. Cells were washed once with Luria broth and resuspended in 1 ml Luria broth. 2 µl of 10 mM of NBD-(linezolid-7-nitrobenz-2-oxa- 1,3-diazol-4-yl)-amino-D-alanine (NADA) (ThermoFisher) was added to the resuspension. Cells were incubated with NADA at 37° C for 30 minutes. Following incubation, cells were washed once and fixed with 1x phosphate buffered saline containing a (1:10) solution of 16% paraformaldehyde.

Microscopy: The microscopy was done as described previously[197]. Briefly, Fixed cells were immobilized on agarose pads and imaged using an inverted Nikon EclipseTi-2 widefield epifluorescence microscope equipped with a Photometrics Prime 95B camera and a Plan Apo 100 m X 1.45 numerical aperture lens objective. Green fluorescence and red fluorescence images were taken using a filter cube with a 470/40 nm or 560/40 nm excitation filters and 632/60 or 535/50 emission filters, respectively. Images were captured using NIS Elements software.

Image analysis: Images were analyzed by Image Fiji[198]. All images were minimally processed (substract background and brightness/contrast adjusted uniformly across all fluorescent images) and pseudo colored (a cyan lookup table was chosen to NADA images). Cells shape (length, area, width and fluorescence intensities) were quantified in MicrobeJ[199] and data were plotted in Prism 9 (GraphPad 9.2.0). Total 300 cells were analyzed for each experiment and independently replicated three times and one representative data was reported in the quantification.

Construction of genetic mutants: The *nagZ, mreB, rodA ,ampD, mpL, pbp2* mutants were constructed as, using one step recombination-mediated genetic engineering[200]. Briefly, a kanamycin resistance cassette flanked by FLP recombination target (FRT) sites was PCR amplified from the pKD4 plasmid using primers containing 125-bp flanking regions of homology

to the gene of interest. The resulting linear PCR product was then transformed via electroporation into *Ab str*ains ATCC 17978 expressing pRECAb (pAT03). Transformants were recovered in Luria broth and plated on LB agar supplemented with 10 mg/L kanamycin. All genetic mutants were confirmed by PCR. Following isolation of genetic mutants, the pMMB67EH::RECAb Tet^R plasmid was removed as described previously. Isolated mutants were grown on LB agar supplemented with 2 mM nickel (II) chloride (NiCl2) and replica plated on LB agar supplemented with kanamycin or tetracycline. Loss of pMMB67EH:RECAb Tet^R plasmid in mutants susceptible to tetracycline and resistant to kanamycin were confirmed using PCR. To excise chromosomal insertion of the kanamycin resistance cassette, cure mutants were transformed with pMMB67EH carrying the FLP recombinase (pAT08) and plated on LB agar supplemented with tetracycline and 2mM Isopropyl β-d-1thiogalactopyranoside (IPTG) to induce expression of FLP recombinase. Successful excision of the kanamycin resistance cassette was confirmed using colony PCR.

Construction of AmpD, ldtK and MurA complementation vectors: AmpD and ElsL complementation vectors were constructed similarly with slight alterations. The AmpD (A1S_0045) and ElsL (A1S_0685) coding sequences were amplified from *Ab* ATCC 17978 cDNA and cloned into BamHI and SalI restriction sites in the pMMB67EHknR plasmid. The resulting pAmpD and ElsL. plasmids were transformed into the respective mutant and induced with 2 mM IPTG for complementation.

Time-dependent killing assays: Meropenem killing experiments were performed as previously described[130]. Wild type, mutant and complementation strains were grown overnight in Luria broth at 37°C. The following day, overnight cultures were back diluted 1:10 in fresh, prewarmed BHI broth containing meropenem or an equivalent volume of water. Diluted BHI cultures were then incubated at 37°C. At 0, 6, 12, and 24 h, each sample was diluted 4-fold in blank BHI, and

the optical density (OD_{600}) was measured. At each time point, cells were serially diluted 10-fold in fresh BHI broth and 5 μL of each serial dilution was spot-plated. Spot-plates were imaged next day. Each experiment was independently replicated three times, and one representative dataset was reported.

Results:

Chromosomally encoded beta-lactamases do not impact Ab survival during meropenem treatment: We previously showed that PG recycling pathway promote the bacterial fitness during meropenem treatment. Based on studies in *Pseudomonas aeruginosa* it is possible that disruption of PG recycling could directly active expression of a carbapenemase to promote survival during treatment [201]. When LTs cleaves the glycosidic linkages between the disaccharide subunits within PG, it yields a soluble 1,6-anhydro-*N*-acetyl-ß-D-muramyl (anhMurNAc) product that can be recycled to promote new PG biosynthesis, PG remodeling or catabolism [74]. Anhydromuropeptides can be transported from the periplasm to through the inner membrane via AmpG [74],[202]. It was previously shown that *Pseudomonas aeruginosa*, *Salmonella enterica* and other Enterobacteriaceae induce expression of a class C beta-lactamases during meropenem treatment [203],[204]. *Ab* encodes two chromosomal beta-lactamases, class D OXA-51 (bla_{OXA-51}) and class C ADC-26 (bla_{ADC})[205]. bla_{OXA-51} encodes a putative carbapenemase and bla_{ADC} encodes a putative cephalosporinase[206], [207]. Previous analysis showed that neither of these genes were induced during meropenem treatment, so we asked if either of these beta-lactamases promotes bacterial fitness during meropenem treatment. We constructed bla_{OXA-51} (A1S_1517) and bla_{ADC} (A1S_2367) mutants, and treated each with high levels (10 μg/mL; 62.5-fold above MIC) of meropenem alongside wild-type. After 24 hr cells were plated on LB agar to determine survival (**Figure 15A**). Wild type and both beta-lactamase mutants (**Figure 15A**) showed

equivalent killing relative to wild type, suggesting there neither beta-lactamases contributes to survival during meropenem treatment. In addition, N-acetyl-anhydromuramyl-L-alanine amidase (AmpD) was shown to be a negative regulator of beta-lactamases expression [208]. However, △*ampD* was also rapidly killed during meropenem treatment relative to wild type (**Figure 15B**). Together, our data suggests that chromosomally encoded *bla*$_{OXA-51}$ and *bla*$_{ADC}$ do not promote survival during meropenem treatment.

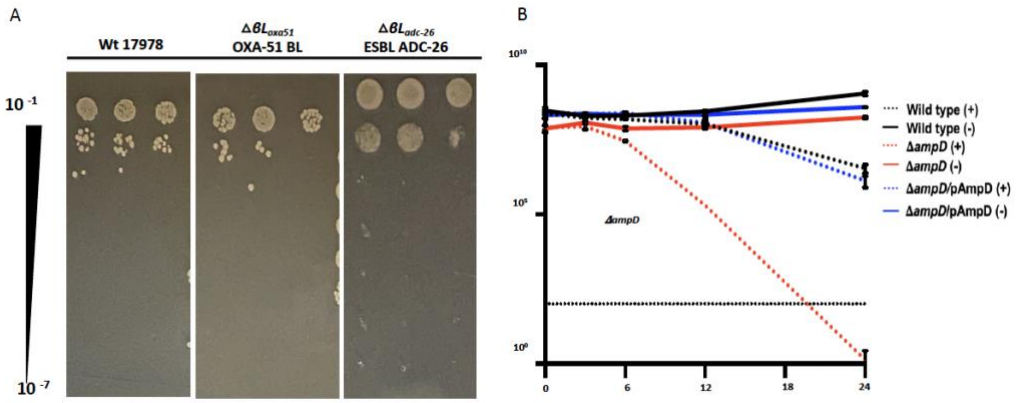

Figure 15: Deletion of either chromosomally encoded beta-lactamase does not impact meropenem-dependent *Ab* survival. (A) Dilution spot assays of *Ab* strains ATCC 17978 (WT) and two beta-lactamases mutants (*ßL*$_{oxa-51}$, *ßL*$_{adc-26}$) after 24 hours of meropenem treatment on BHI agar medium. (B) Meropenem tolerance assay for 24 hours with (+) and without (-) meropenem in Wt, **Δ***ampD* and **Δ***ampD/*pAmpD. Each experiment was independently replicated two times in triplicate, one representative data set was reported.

Deletions in each PG recycling gene promote morphological defects: In *E. coli*, it's been reported that 1,6 anhydroMurNAc-tetrapeptide containing muropeptides are imported into the cytoplasm via AmpG (transmembrane permease). 1,6 anhydroMurNAc tetrapeptide is modified by a series

on enzymes, including the L,D-carboxypeptidase LdcA [209], [210], the beta-hexosaminidase, NagZ [211] and 1,6-anhydro-N-acetylmuramoyl-L-alanine amidase, AmpD [193] to produce 1,6 anhydrotripeptide (TriAnh), 1,6-anhMurNAc, and tripeptides respectively. The tripeptides can be further degraded into individual amino acids to provide nutrients or energy source for the cell. Alternatively UDP-N-acetylmuramate—L-alanyl-γ-D-glutamyl-meso-2,6-diaminoheptanedioate ligase, MpL [175], ligated tripeptides to UDP-MurNAc in the recycling pathway to for UDP-MurNAc-tripeptide which can act as an intermediate in the *de novo* PG synthesis pathway [77],[212]. UDP-MurNAc-tripeptide acts a s a substrate for UDP-N-acetylmuramopyl-tripeptide-D-alanyl-D-alaine ligase, MurF [213] to form lipidII. In our previous study, we found ElsL and AmpD contributed to meropenem tolerance in *Ab* [49]. Deletion of either genes also showed a morphology defect characterized by sphere/round shaped cells (**Figure 16 B & C**).

Next, we asked if all of the genes involved in PG recycling share the same morphological defect when deleted. We also made isogenic mutants *ampG*, *nagZ* and *mpL* and found that all PG recycling mutants were characterized by population with rounded cell morphology (**Figure 16 D-F).** Furthermore, in △*ampG* we found that some mutants had rounded cell morphologies and others had coccobacilli morphologies (**Figure 16 F**). We used whole-genome sequencing to identify a suppressor mutant in Y41 position of *adeN* in all the *ampG* mutants. AdeN is a negative regulator of the genes encoding the AdeIJK efflux system, a mechanism that actively works to efflux antibiotics from the cell and promotes bacterial fitness [214], [101], [100]. While it isn't obvious how the efflux system restores the cell shape defects, it is work noting beta-lactams, which closely mimic the D-ala, D-ala peptides, are a substrate.

We also calculated the surface area, and width of wild type and mutants (**Figure 16G- H**), and all mutants showed an increased surface area and width relative to wild type. Together these data

suggest that the PG recycling pathway is important for cell shape, where deletion of each isogenic gene induced round-shaped cells suggesting PG recycling contributes to PG elongation.

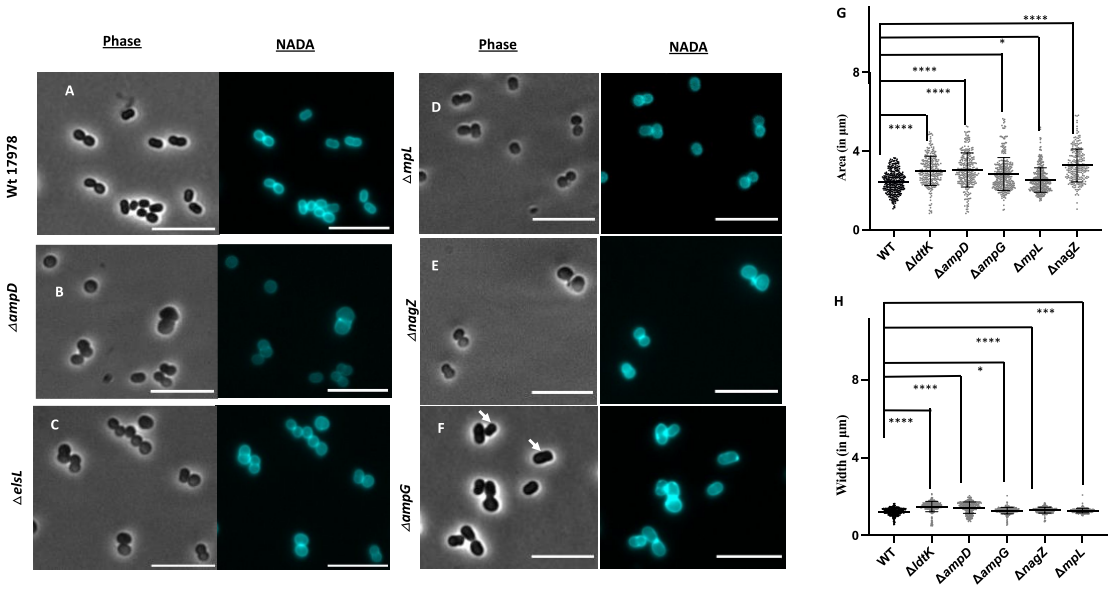

Figure 16: Microscopy of *Ab* 17978 WT and PG recycling mutants (*ldtK, ampD, mpL, nagZ, ampG*) in logarithmic phase. Phase and fluorescence microscopy of wild-type (WT) 17978 (A), *⊿ ampD* (B), *△elsL* (C), *△mpL* (D), *△nagZ* (E) and *△ampG* (F) cells. Cells in mid-logarithmic growth were labeled with NADA, Scale bar 10 µM, White arrow indicates rod shaped cells. G) Quantification of surface area of wild -type and the mutants (*n*=300) in logarithm phase, H) Quantification of width of wild -type and mutants (*n*=300) in logarithm phase was calculated through ImageJ. Each experiment was replicated three times and one representative data was reported. Each dot on the graph represents one cell. Unpaired t-test was done (P value <0.05) was done in wild -type vs PG recycling mutants for determining statistical significance.

PG recycling and rod-complex mutants are phenotypically identical: Bacterial cells continuously undergo elongation and division to proliferate. Two distinct protein complexes coordinate either rod-dependent PG biosynthesis (elongasome) and septal-dependent PG biosynthesis (divisome)

[215]. The Rod complex coordinates elongation, which consists of several proteins such as MreB, a transmembrane protein RodA, and a PG synthesis enzyme penicillin-binding protein PBP2. In *E. coli*, RodA is thought to be essential [216] and both RodA and PBP2 showed septation specific counterparts encoded by ftsW. MreB is an actin-like homolog that forms antiparallel filaments that bind the membrane [217],[218]. It plays a critical role in determining bacterial shape[219].With the association of MreB, the RodA-PBP2 complex insert new hoops of PG at dispersed sites throughout the cell cylinder and promote its elongation[220]. In *Ab*, a conserved zinc-binding site in PBP2 is required for elongasome-directed bacterial cell shape [221]. Although a complete picture of other rod system core components is still lacking in *Ab*. To visualize the morphological defects when Rod-dependent PG insertion is defective, I deleted *rodA, pbp2* and mreB, which were all thought to be essential in rod-shaped bacteria. Phase and fluorescence microscopy showed that △*rodA*, △*pbp2* and △*mreB* (**Figure 17 D-F**) produced spherical cells, suggesting elongation defects. Interestingly, the morphology of genes associated with rod complex is identical to other PG recycling mutants, including *ampD* and *elsL* (**Figure 17 B&C**). We measured the width, length, and surface area of the all the mutants associated with PG recycling and the PG elongation. All mutants show higher width and surface area relative to the wild type (**Figure 17 G**), and there are no appreciable changes between recycling mutants and elongation mutants (*elsL/ampD vs pbp2, rodA or mreB*). Overall, these data suggest a link between the PG recycling and cell elongation. Perhaps PG recycling provides lipid II substrates needed for cell elongation in *Ab*.

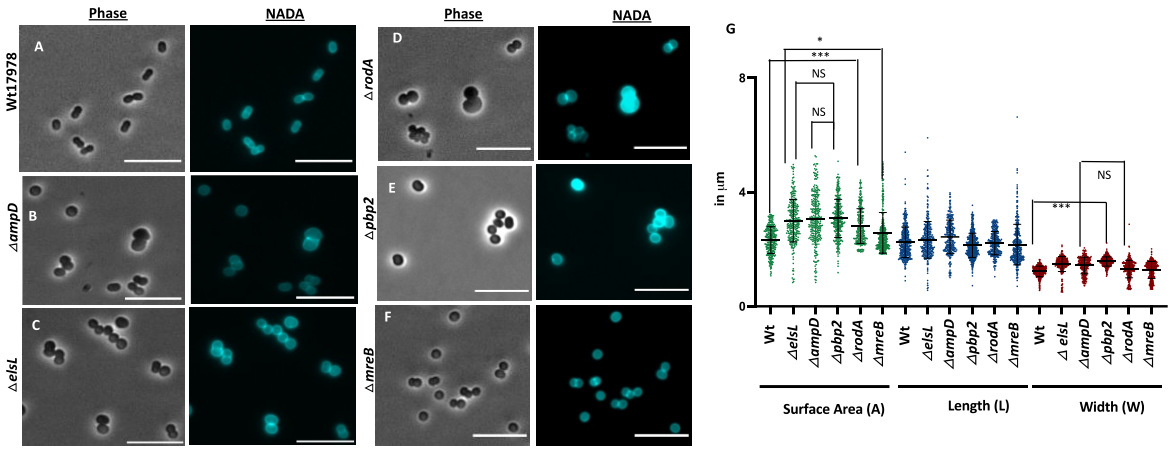

Figure 17: Microscopy of *Ab* 17978 WT and mutants involved in PG elongation (△ *rodA*,△ *pbp2*, △*mreB*) in logarithmic growth phase. Phase and fluorescence microscopy of wil*d*-type (WT) 17978 (A), △*ampD (B), △ldtK (C)* △*rodA* (D), △*pbp2* (E) and △*mreB* (F) cells. Cells in mid-logarithmic growth were labeled with NADA. G) Quantification of surface area, length and of WT and the mutants (n=300) in logarithm phase, H) Quantification of width of WT and mutants (n=300) in logarithm phase was calculated through ImageJ. Each experiment was replicated three times and one representative data was reported. Each dot on the graph represents one cell. Significance was determined using an unpaired t-test ($P < 0.05$) in WT vs mutants.

PG recycling is required for Rod dependent PG biosynthesis: Based on our data (**Figure 17**) and other studies [210] we hypothesized that the pool of lipid II may be limited in *Ab*, so competition for the substrate pool is probably more efficiently used by the divisome, with little activity by the Rod complex to produce the wild type coccobacilli morphology. We know from previous data that mutations that slow division promote Rod-activity, presumably by increasing the lipid II pool [197], [222]. In contrast mutations in PG recycling that presumably reduce inhibit the lipid II pool may not provide substrate for the Rod-complex.

Since there is a morphological similarity between the genes associated PG recycling and cell elongation, we reasoned that cell elongation is impacting PG recycling either directly or indirectly. Previously it is reported the *elsL* contributes to rod activity and *elsL* mutation produces an accumulation of tetrapeptides which is toxic [182],[49],[197].

To understand the missing link, here we overexpressed the PG recycling genes to the wild type. We predict that if PG recycling contributes directly to elongation, overexpression of PG recycling will produce elongated cell relative to wild type. First, we overexpressed AmpD and ElsL to the wild type (**Figure 18, A-C**). While the changes were not grossly obvious, there were slight differences, where the cells were narrow and showed reduced surface area, both indicative of increased Rod-dependent PG activity. Together, these findings suggest that PG recycling may be required for Rod-dependent PG biosynthesis in *Ab*.

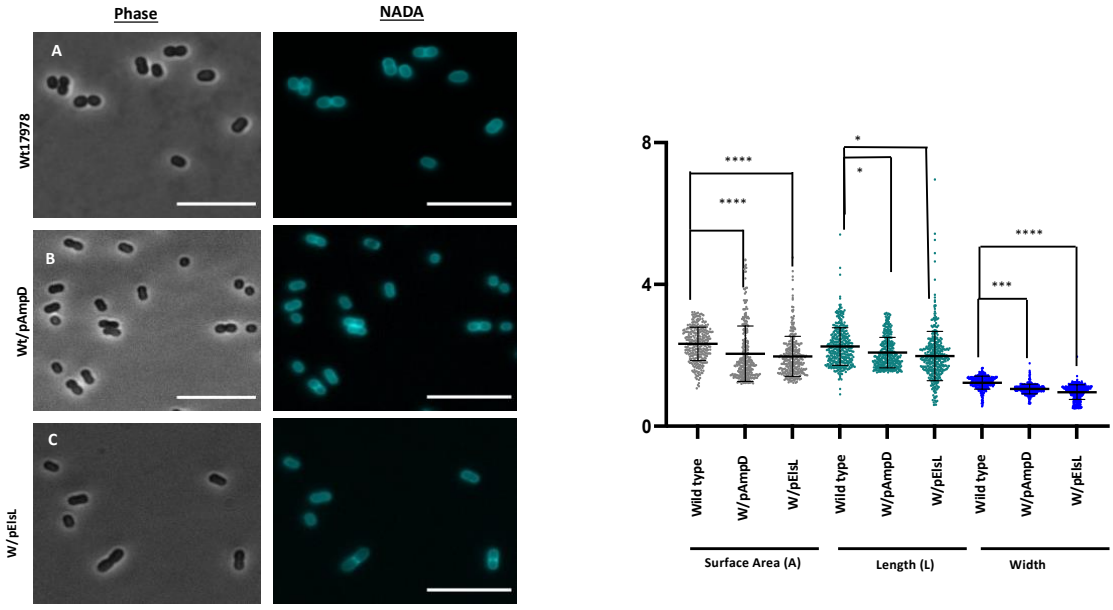

Figure 18: Microscopic observation of *Ab* 17978 WT, W/pAmpD, W/pLdtK, in logarithmic growth phase. Phase and fluorescence microscopy of wild-type (WT) 17978 (A), W/pAmpD (B), W/pElsL (C) cells. Cells in mid-logarithmic growth were labeled with NADA. D) Quantification

of surface area (A), Length (L) and Width of (W) of Wt and W/pAmpD,W/pLdtK, W/pMurA (n=300) in logarithm phase were calculated through ImageJ. Each experiment was replicated three time and one representative data was reported. Each dot on the graph represents one cell.

Discussion:

While studying the beta-lactamase induction and PG recycling pathway during meropenem treatment in *Ab*, we found that defects in PG recycling impact elongation (**Figure 17**)**.** Since all genes tested in the PG recycling pathway were required for rod-shaped morphology, it implies that limiting the lipid II pool inhibit Rod-dependent PG elongation in *Ab*.

The Rod complex and the divisome complex utilize the same lipid precursor to polymerize PG during growth [223], [224]. Disruption to the major *Ab* PG synthase PBP1A delays septation [222], [197] and produces elongated cells. PBP1A is enriched at the septum where it could directly help recruit lipid II through increased affinity relative to the Rod-complex. Substrate competition between the two synthases (Rod complex and divisome) , as previously described [225], [226] could provide a possible explanation as to why *Ab* forms coccobacilli, with minimal axial PG biosynthesis and increased septation if the divisome complex can recruit lipid II more efficiently.

We hypothesized that disruption of PG recycling may limit the lipid II pool to reduce substrate for Rod-complex activity, while not affecting division. Alternatively, overexpressing PG recycling genes, appeared to increase Rod-dependent PG synthesis (cells were narrower and more had a smaller surface area) (**Fig 18E**). While the mechanistic basis for competition between cell division and elongation has not been determined, endopeptidase activation impaired the cell division in *E. coli* that promotes cell elongation [220]. Another study by Lai *et al*., 2017 reported that overproduction of an endopeptidase is required to enhance PG synthesis by the aPBPs [227].

Therefore, division defects in endopeptidase overproducing cells increased competition for substrate towards the divisome complex to promote axial PG biosynthesis. Interestingly, unpublished work from our laboratory showed that the D,D-carboxypeptidase, PBP5, which is likely part of the Rod-complex cleaves the terminal D-alanine on pentapeptide subunits to yield tetrapeptides, also caused wild type coccobacilli cells to grow as spheres. Together, these studies suggest that division may increase when cell elongation is inhibited, possibly perturbing the competition for lipid II substrate between the elogasome and divisome synthases.

In addition, our results clearly demonstrated that PG recycling is critical in controlling elongation activities in during *Ab* growth. Overexpression of ElsL and AmpD may help restore Rod-dependent PG biosynthesis (**Figure 18**). A recent study showed that MurA-F, suppress the toxicity in *E. coli* and *Vibrio cholerae* and help to correct both biosynthesis and cross-linking issues [228]. Next, we need to explore the PG recycling requirement in *Ab* when we increase the total pool of lipid II. We can overexpress MurA to increase the lipid II pool and test if PG recycling is still important for cell elongation. Overall, our data suggests that *de novo* biosynthesis pathway is critical *in Ab* growth, and the PG precursor composition can determine a cell's preference for lateral versus septal cell wall growth.

Unlike rod shaped bacteria such as *E. coli* and *Bacillus subtilis*[217], *mreB, pbp2* and *rodA* are found to be nonessential for viability in *Ab*. In the rod-shaped bacteria, RodA-PBP2 complex forms the core PG synthase of the Rod system and activation of PBP2 suppress the growth defect of mreC hypomorphs [217]. However, in *Ab* it is unclear, how the enzymatic activity of RodA-PBP2 complex is coordinated when there is a mutation in one gene. It is also important to know if there are any additional proteins MreC, MreD and RodZ working when RodA-PBP2 is being affected.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

The principal objective of this work was to determine the mechanisms that contribute to antimicrobial treatment failure in clinically significant Gram-negative bacterial species *Ab* to prevent the spread of antimicrobial resistance crisis. While factors that precede resistance have been already known, the key factors that led to tolerance remain understudied. In this study, we identified the genetic determinants that are responsible for carbapenem tolerance in *Ab*. We also defined the enzymatic characteristic of two PG maintenance genes, Penicillin-binding protein PBP7 (encoded by *pbpG*) and ElsL (also known as LdtK), required for cell wall integrity and PG recycling. We have also characterized how PG recycling impacted the rod/elongation activity in *Ab*. Overall, these works show the mechanistic basis for envelope remodeling by the genes associated with OM integrity and PG maintenance in response to PG defects by cell wall-acting antibiotics. Thus, a better understanding of PG recycling and OM integrity would provide us with grater insights into combatting to antibiotic tolerance and resistance.

Carbapenem antibiotic has a broad-spectrum activity to treat multidrug-resistant Gram-negative bacterial infections [37], [61], [229], [230]. However, the increasing trend of carbapenem resistance *Ab* (CRAB) infections has become a global health concern due to the scarcity of antimicrobial therapeutics and the rapid rise of the resistance phenomenon [61],[18]. Along with the resistance, tolerance also contributes to treatment failure by carrying a reservoir of adaptable cells for an extended period during antimicrobial therapy. These tolerant populations are called the dormant contributors to treatment failure due to no appreciable changes in MIC and their not being detected by conventional antimicrobial susceptibility testing methods [231]. Therefore, they directly and indirectly contribute to developing resistance by horizontal gene transfer or mutations.

It suggests that they pose a serious concern the spread of bacterial infections. Here, in chapter 3, we identified both susceptible lab-adapted and recently isolated clinical strains of *Ab* survive up to 24 hours of lethal levels of meropenem. These indicated that susceptible strains of *Ab* exhibit high tolerance to meropenem. Furthermore, we found that spheroplast production is mediated by the tolerant cell, and upon removal of the antibiotic, these cells can revert to their normal coccobacilli cell morphology. Prior studies on Enterobacteriaceae also showed spheroplast production during meropenem treatment [53]; however, how bacteria survive and maintain internal turgor pressure without a cell wall has not been characterized. In Chapter 3, we first reasoned that elucidating genetic and molecular factors during meropenem treatment will provide novel insight into the incredible ability of this pathogen and help uncover new targets to slow the evolution of resistance. Based on transcriptome analysis (Rna-seq) of meropenem-induced spheroplasts compared to untreated *Ab* cells, we found upregulation of genes that are associated with the efflux system of beta-lactam suggesting bacteria continuously increased efflux to pump out the antibiotic from the periplasm. In addition, we found downregulations of genes encoded outer membrane porins that limit the influx of meropenem into the cell. Moreover, we found an upregulation of several LTs during meropenem treatment that suggests that, beta-lactam inhibits the cell wall biosynthesis by blocking the transpeptidase activity of penicillin-binding proteins (PBPs) that is further cleaved by LTs to mitigate the periplasmic crowding and increase periplasmic PG turnover [75], [232]. These PG turnover fragments are further transported into the cytoplasm for recycling to build *de novo* PG synthesis. Overall, our transcriptome data indicates that PG remodeling plays a crucial role during meropenem-induced spheroplast formation and suggests that induction of LTs are critical for maintaining cell osmolarity during stress condition. Subsequently, transposon insertion sequencing (Tn-seq) revealed the fitness factors involved in *Ab* by comparing insertional

mutants recovered from treated and untreated cells. We screened genes contributing to outer membrane stability *ompA, lpxM,* as well as PG recycling *pbpG, elsL* and *ampD, which* are required for fitness during meropenem treatment. Overall, our data suggests both the outer membrane integrity and PG recycling genes work together and maintain cell homeostasis when the cell wall is disrupted. In addition, in this chapter, we have characterized the enzymatic characteristics of two genetically important determinants during meropenem treatment. We identified that PBP7, which is a penicillin-binding protein, is a low molecular weight PBP encoded by *pbpG* that has both DD-carboxypeptidase and endopeptidase activity. At the same time, ElsL represents the first known YkuD-domain-containing protein with LD-carboxypeptidase and is located in the cytoplasm. PBP7, coupled with the LTs, produces PG turnover products that are further processed by the cytoplasmic recycling genes *elsL* and *ampD*. Together, our data provide evidence to identify novel factors that can be a potential target to inhibit tolerance and slow down the spread of resistance. This study also lets us know that the outer membrane provides structural integrity when cell wall is messed up. However, it is still unknown how PG recycling promotes bacterial survivability during stress conditions. Moreover, our study suggests that combinatorial therapies targeting cell wall biosynthesis and recycling pathways will be an effective treatment option. Since *Ab* is intrinsically resistant to Fosfomycin (inhibits cell wall by inactivating UDP-N-acetylglucosamine-3-o-enolpyruvultransferase) [233]. Future study is needed for the development of new therapeutics that target the both PG synthesis and PG recycling. Also, an *in vivo* animal model study with the recycling and OM integrity maintenance mutants will reveal more knowledge to understand how this recycling pathway is critical for bacterial survival in host cells.

In Chapter 4, I investigated how PG recycling promotes bacterial fitness. Prior studies showed that the PG recycling pathway is critical for expressing AmpC beta-lactamases in the

presence of carbapenem in many Gram-negative pathogens [204],[80]. Since *Ab* encoded two beta-lactamases on its chromosome, blaADC (AmpC) and blaOXA-51[234] in the presence of carbapenem, here, we investigated if induction of beta-lactamases is also vital by cell wall recycling during antibiotic tolerance in *Ab*. We found that beta-lactamase induction is not required for extended survival, and the *de novo* recycling pathway is critical to promoting bacterial survival. Most importantly, in Chapter 4, we found a correlation between cell elongation and PG recycling. Deficiency in PG recycling genes produces sphere-shaped morphology, which is similar to when the rod system has malfunctioned. Overall, our data suggests that, most likely, competition between PG divisome and elongation complex for cell wall precursor (lipid II), and activity of PG recycling antagonizes cell division and promotes cell elongation. Future studies will provide us with more evidence of how cell wall recycling is related to cell wall elongation.

In addition, overexpression of AmpD and ElsL slightly induce cell surface area and width. Overexpression of *de novo* biosynthesis MurA may provide us more knowledge if PG recycling impacts cell elongation. A more detailed oriented analysis is needed to disclose the role of the recycling pathway in *Ab*.

In conclusion, this study is unique in *Ab*, and our findings provide evidence of how the OM integrity and PG maintenance genes working together for maintaining cell envelope stability and promote bacterial survivability. Here, we propose the potential targets that need to be treated in the future to slow down the spread of resistance. Overall, it emphasizes that there is an urgent need for the development of novel therapeutic strategies.

References:

[1] I. Levin-Reisman, A. Brauner, I. Ronin, and N. Q. Balaban, "Epistasis between antibiotic tolerance, persistence, and resistance mutations," *Proc. Natl. Acad. Sci.*, vol. 116, no. 29, pp. 14734–14739, Jul. 2019, doi: 10.1073/pnas.1906169116.

[2] J. Davies and D. Davies, "Origins and Evolution of Antibiotic Resistance," *Microbiol. Mol. Biol. Rev. MMBR*, vol. 74, no. 3, pp. 417–433, Sep. 2010, doi: 10.1128/MMBR.00016-10.

[3] M. Huemer, S. Mairpady Shambat, S. D. Brugger, and A. S. Zinkernagel, "Antibiotic resistance and persistence—Implications for human health and treatment perspectives," *EMBO Rep.*, vol. 21, no. 12, p. e51034, Dec. 2020, doi: 10.15252/embr.202051034.

[4] C. L. Ventola, "The Antibiotic Resistance Crisis," *Pharm. Ther.*, vol. 40, no. 4, pp. 277–283, Apr. 2015, Accessed: Sep. 12, 2023. [Online]. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4378521/

[5] CDC, "National Infection & Death Estimates for AR," Centers for Disease Control and Prevention. Accessed: Sep. 13, 2023. [Online]. Available: https://www.cdc.gov/drugresistance/national-estimates.html

[6] I. Santi, P. Manfredi, E. Maffei, A. Egli, and U. Jenal, "Evolution of Antibiotic Tolerance Shapes Resistance Development in Chronic Pseudomonas aeruginosa Infections," *mBio*, vol. 12, no. 1, p. 10.1128/mbio.03482-20, Feb. 2021, doi: 10.1128/mbio.03482-20.

[7] A. Brauner, N. Shoresh, O. Fridman, and N. Q. Balaban, "An Experimental Framework for Quantifying Bacterial Tolerance," *Biophys. J.*, vol. 112, no. 12, pp. 2664–2671, Jun. 2017, doi: 10.1016/j.bpj.2017.05.014.

[8] L. F. Westblade, J. Errington, and T. Dörr, "Antibiotic tolerance," *PLOS Pathog.*, vol. 16, no. 10, p. e1008892, Oct. 2020, doi: 10.1371/journal.ppat.1008892.

[9] A. J. Lopatkin, T. A. Sysoeva, and L. You, "Dissecting the effects of antibiotics on horizontal gene transfer: Analysis suggests a critical role of selection dynamics," *BioEssays News Rev. Mol. Cell. Dev. Biol.*, vol. 38, no. 12, pp. 1283–1292, Dec. 2016, doi: 10.1002/bies.201600133.

[10] Y. Liu, K. Yang, H. Zhang, Y. Jia, and Z. Wang, "Combating Antibiotic Tolerance Through Activating Bacterial Metabolism," *Front. Microbiol.*, vol. 11, p. 577564, Oct. 2020, doi: 10.3389/fmicb.2020.577564.

[11] A. Tilanus and G. Drusano, "Optimizing the Use of Beta-Lactam Antibiotics in Clinical Practice: A Test of Time," *Open Forum Infect. Dis.*, vol. 10, no. 7, p. ofad305, Jul. 2023, doi: 10.1093/ofid/ofad305.

[12] M. Mora-Ochomogo and C. T. Lohans, "β-Lactam antibiotic targets and resistance mechanisms: from covalent inhibitors to substrates," *RSC Med. Chem.*, vol. 12, no. 10, pp. 1623–1639, doi: 10.1039/d1md00200g.

[13] S. Garde, P. K. Chodisetti, and M. Reddy, "Peptidoglycan: Structure, Synthesis, and Regulation," *EcoSal Plus*, vol. 9, no. 2, Jan. 2021, doi: 10.1128/ecosalplus.ESP-0010-2020.

[14] O. Irazoki, S. B. Hernandez, and F. Cava, "Peptidoglycan Muropeptides: Release, Perception, and Functions as Signaling Molecules," *Front. Microbiol.*, vol. 10, 2019,

Accessed: Sep. 23, 2023. [Online]. Available: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00500
[15] Y. Ye, Y. Kong, J. Ma, and G. Shi, "Carbapenem-Resistant Gram-Negative Bacteria-Related Healthcare-Associated Ventriculitis and Meningitis: Antimicrobial Resistance of the Pathogens, Treatment, and Outcome," *Microbiol. Spectr.*, vol. 10, no. 3, pp. e00253-22, doi: 10.1128/spectrum.00253-22.
[16] J. Lu *et al.*, "Effectiveness of a double-carbapenem combinations against carbapenem-resistant Gram-negative bacteria," *Saudi Pharm. J. SPJ*, vol. 30, no. 6, pp. 849–855, Jun. 2022, doi: 10.1016/j.jsps.2022.03.007.
[17] A. Michalopoulos and M. E. Falagas, "Treatment of Acinetobacter infections," *Expert Opin. Pharmacother.*, vol. 11, no. 5, pp. 779–788, Apr. 2010, doi: 10.1517/14656561003596350.
[18] "Carbapenem-resistant Acinetobacter baumannii (CRAB): An urgent public health threat in United States healthcare facilities | A.R. & Patient Safety Portal." Accessed: Sep. 21, 2023. [Online]. Available: https://arpsp.cdc.gov/story/cra-urgent-public-health-threat
[19] C. Ayoub Moubareck and D. Hammoudi Halat, "Insights into Acinetobacter baumannii: A Review of Microbiological, Virulence, and Resistance Traits in a Threatening Nosocomial Pathogen," *Antibiotics*, vol. 9, no. 3, p. 119, Mar. 2020, doi: 10.3390/antibiotics9030119.
[20] G. Marioni *et al.*, "Deep neck abscess due to Acinetobacter baumannii infection," *Am. J. Otolaryngol.*, vol. 31, no. 4, pp. 304–307, 2010, doi: 10.1016/j.amjoto.2009.02.018.
[21] J. M. Boll *et al.*, "Reinforcing Lipid A Acylation on the Cell Surface of Acinetobacter baumannii Promotes Cationic Antimicrobial Peptide Resistance and Desiccation Survival," *mBio*, vol. 6, no. 3, p. 10.1128/mbio.00478-15, May 2015, doi: 10.1128/mbio.00478-15.
[22] N. Gupta, K. Angadi, and S. Jadhav, "Molecular Characterization of Carbapenem-Resistant Acinetobacter baumannii with Special Reference to Carbapenemases: A Systematic Review," *Infect. Drug Resist.*, vol. 15, pp. 7631–7650, Dec. 2022, doi: 10.2147/IDR.S386641.
[23] Y. Oda *et al.*, "CsrA-Controlled Proteins Reveal New Dimensions of Acinetobacter baumannii Desiccation Tolerance," *J. Bacteriol.*, vol. 204, no. 4, pp. e00479-21, Mar. 2022, doi: 10.1128/jb.00479-21.
[24] "The origin and distribution of microzymes (bacteria) in water, and the circumstances which determine their existence in the tissues and liquids of the living body / by Dr. Burdon-Sanderson.," Wellcome Collection. Accessed: Oct. 01, 2023. [Online]. Available: https://wellcomecollection.org/works/zemuyrtf/items
[25] S. Selwyn, "Pioneer work on the 'penicillin phenomenon', 1870–1876," *J. Antimicrob. Chemother.*, vol. 5, no. 3, pp. 249–255, May 1979, doi: 10.1093/jac/5.3.249.
[26] K.-F. Kong, L. Schneper, and K. Mathee, "Beta-lactam Antibiotics: From Antibiosis to Resistance and Bacteriology," *APMIS Acta Pathol. Microbiol. Immunol. Scand.*, vol. 118, no. 1, pp. 1–36, Jan. 2010, doi: 10.1111/j.1600-0463.2009.02563.x.
[27] J. J. Leisner, "The Diverse Search for Synthetic, Semisynthetic and Natural Product Antibiotics From the 1940s and Up to 1960 Exemplified by a Small Pharmaceutical Player," *Front. Microbiol.*, vol. 11, 2020, Accessed: Oct. 01, 2023. [Online]. Available: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00976
[28] "Beta Lactam - an overview | ScienceDirect Topics." Accessed: Oct. 01, 2023. [Online]. Available: https://www.sciencedirect.com/topics/medicine-and-dentistry/beta-lactam

[29] A. Chen, “Beta-lactam Antibiotics: The Past, Present, and Future,” NHSJS. Accessed: Oct. 01, 2023. [Online]. Available: https://nhsjs.com/2021/beta-lactam-antibiotics-the-past-present-and-future/
[30] N. Pandey and M. Cascella, “Beta-Lactam Antibiotics,” in *StatPearls*, Treasure Island (FL): StatPearls Publishing, 2023. Accessed: Oct. 01, 2023. [Online]. Available: http://www.ncbi.nlm.nih.gov/books/NBK545311/
[31] J. Turner *et al.*, “The Chemical Relationship Among Beta-Lactam Antibiotics and Potential Impacts on Reactivity and Decomposition,” *Front. Microbiol.*, vol. 13, 2022, Accessed: Oct. 01, 2023. [Online]. Available: https://www.frontiersin.org/articles/10.3389/fmicb.2022.807955
[32] K. Bush and P. A. Bradford, “β-Lactams and β-Lactamase Inhibitors: An Overview,” *Cold Spring Harb. Perspect. Med.*, vol. 6, no. 8, p. a025247, Aug. 2016, doi: 10.1101/cshperspect.a025247.
[33] E. Sauvage, F. Kerff, M. Terrak, J. A. Ayala, and P. Charlier, “The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis,” *FEMS Microbiol. Rev.*, vol. 32, no. 2, pp. 234–258, Mar. 2008, doi: 10.1111/j.1574-6976.2008.00105.x.
[34] E. Sauvage and M. Terrak, “Glycosyltransferases and Transpeptidases/Penicillin-Binding Proteins: Valuable Targets for New Antibacterials,” *Antibiotics*, vol. 5, no. 1, p. 12, Feb. 2016, doi: 10.3390/antibiotics5010012.
[35] BarryG. Hall, StephenJ. Salipante, and M. Barlow, “Independent Origins of Subgroup Bl+B2 and Subgroup B3Metallo-?-Lactamases,” *J. Mol. Evol.*, vol. 59, no. 1, Jul. 2004, doi: 10.1007/s00239-003-2572-9.
[36] E. K. Sinner, M. S. Lichstrahl, R. Li, D. R. Marous, and C. A. Townsend, “Methylations in complex carbapenem biosynthesis are catalyzed by a single cobalamin-dependent radical *S* -adenosylmethionine enzyme,” *Chem. Commun.*, vol. 55, no. 99, pp. 14934–14937, 2019, doi: 10.1039/C9CC07197K.
[37] J. S. Bradley, J. Garau, H. Lode, K. V. I. Rolston, S. E. Wilson, and J. P. Quinn, “Carbapenems in clinical practice: a guide to their use in serious infection,” *Int. J. Antimicrob. Agents*, vol. 11, no. 2, pp. 93–100, Feb. 1999, doi: 10.1016/S0924-8579(98)00094-6.
[38] G. G. Zhanel, A. E. Simor, L. Vercaigne, and L. Mandell, “Imipenem and meropenem: Comparison of in vitro activity, pharmacokinetics, clinical trials and adverse effects,” *Can. J. Infect. Dis.*, vol. 9, no. 4, pp. 215–228, 1998, Accessed: Oct. 02, 2023. [Online]. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3250889/
[39] J. Clardy, M. Fischbach, and C. Currie, “The natural history of antibiotics,” *Curr. Biol. CB*, vol. 19, no. 11, pp. R437–R441, Jun. 2009, doi: 10.1016/j.cub.2009.04.001.
[40] R. I. Aminov, “A Brief History of the Antibiotic Era: Lessons Learned and Challenges for the Future,” *Front. Microbiol.*, vol. 1, p. 134, Dec. 2010, doi: 10.3389/fmicb.2010.00134.
[41] E. F. Haney and R. E. W. Hancock, “Addressing Antibiotic Failure—Beyond Genetically Encoded Antimicrobial Resistance,” *Front. Drug Discov.*, vol. 2, 2022, Accessed: Sep. 12, 2023. [Online]. Available: https://www.frontiersin.org/articles/10.3389/fddsv.2022.892975
[42] T. M. Uddin *et al.*, “Antibiotic resistance in microbes: History, mechanisms, therapeutic strategies and future prospects,” *J. Infect. Public Health*, vol. 14, no. 12, pp. 1750–1766, Dec. 2021, doi: 10.1016/j.jiph.2021.10.020.
[43] “Antibiotic resistance.” Accessed: Oct. 02, 2023. [Online]. Available: https://www.who.int/news-room/fact-sheets/detail/antibiotic-resistance

[44] "Stop the Spread of Superbugs," NIH News in Health. Accessed: Oct. 02, 2023. [Online]. Available: https://newsinhealth.nih.gov/2014/02/stop-spread-superbugs
[45] J. M. Munita and C. A. Arias, "Mechanisms of Antibiotic Resistance," *Microbiol. Spectr.*, vol. 4, no. 2, p. 10.1128/microbiolspec.VMBF-0016–2015, Apr. 2016, doi: 10.1128/microbiolspec.VMBF-0016-2015.
[46] E. M. Darby *et al.*, "Molecular mechanisms of antibiotic resistance revisited," *Nat. Rev. Microbiol.*, vol. 21, no. 5, Art. no. 5, May 2023, doi: 10.1038/s41579-022-00820-y.
[47] Y. N. Chiang, J. R. Penadés, and J. Chen, "Genetic transduction by phages and chromosomal islands: The new and noncanonical," *PLoS Pathog.*, vol. 15, no. 8, p. e1007878, Aug. 2019, doi: 10.1371/journal.ppat.1007878.
[48] D. Sun, K. Jeannot, Y. Xiao, and C. W. Knapp, "Editorial: Horizontal Gene Transfer Mediated Bacterial Antibiotic Resistance," *Front. Microbiol.*, vol. 10, 2019, Accessed: Oct. 02, 2023. [Online]. Available: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01933
[49] N. Islam *et al.*, "Peptidoglycan Recycling Promotes Outer Membrane Integrity and Carbapenem Tolerance in Acinetobacter baumannii," *mBio*, vol. 13, no. 3, pp. e01001-22, Jun. 2022, doi: 10.1128/mbio.01001-22.
[50] T. Dörr, "Understanding tolerance to cell wall–active antibiotics," *Ann. N. Y. Acad. Sci.*, vol. 1496, no. 1, pp. 35–58, 2021, doi: 10.1111/nyas.14541.
[51] I. Levin-Reisman, I. Ronin, O. Gefen, I. Braniss, N. Shoresh, and N. Q. Balaban, "Antibiotic tolerance facilitates the evolution of resistance," *Science*, vol. 355, no. 6327, pp. 826–830, Feb. 2017, doi: 10.1126/science.aaj2191.
[52] C. R. Miller, J. M. Monk, R. Szubin, and A. D. Berti, "Rapid resistance development to three antistaphylococcal therapies in antibiotic-tolerant staphylococcus aureus bacteremia," *PLoS ONE*, vol. 16, no. 10, p. e0258592, Oct. 2021, doi: 10.1371/journal.pone.0258592.
[53] T. Cross *et al.*, "Spheroplast-Mediated Carbapenem Tolerance in Gram-Negative Pathogens," *Antimicrob. Agents Chemother.*, vol. 63, no. 9, pp. e00756-19, Aug. 2019, doi: 10.1128/AAC.00756-19.
[54] A. N. Murtha *et al.*, "High-level carbapenem tolerance requires antibiotic-induced outer membrane modifications," *PLoS Pathog.*, vol. 18, no. 2, p. e1010307, Feb. 2022, doi: 10.1371/journal.ppat.1010307.
[55] S. K. Panda *et al.*, "Recent advances to combat ESKAPE pathogens with special reference to essential oils," *Front. Microbiol.*, vol. 13, 2022, Accessed: Oct. 03, 2023. [Online]. Available: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1029098
[56] H. J. Mea, P. V. C. Yong, and E. H. Wong, "An overview of Acinetobacter baumannii pathogenesis: Motility, adherence and biofilm formation," *Microbiol. Res.*, vol. 247, p. 126722, Jun. 2021, doi: 10.1016/j.micres.2021.126722.
[57] "baumannii implant-associated osteomyelitis: Topics by Science.gov." Accessed: Oct. 03, 2023. [Online]. Available: https://www.science.gov/topicpages/b/baumannii+implant-associated+osteomyelitis
[58] "Acinetobacter baumannii Infections Among Patients at Military Medical Facilities Treating Injured U.S. Service Members, 2002--2004." Accessed: Sep. 21, 2023. [Online]. Available: https://www.cdc.gov/mmwr/preview/mmwrhtml/mm5345a1.htm
[59] C. M. Harding, S. W. Hennon, and M. F. Feldman, "Uncovering the mechanisms of Acinetobacter baumannii virulence," *Nat. Rev. Microbiol.*, vol. 16, no. 2, Art. no. 2, Feb. 2018, doi: 10.1038/nrmicro.2017.148.

[60] G. Meletis, “Carbapenem resistance: overview of the problem and future perspectives,” *Ther. Adv. Infect. Dis.*, vol. 3, no. 1, pp. 15–21, Feb. 2016, doi: 10.1177/2049936115621709.
[61] N. Al-Rashed *et al.*, “Prevalence of Carbapenemases in Carbapenem-Resistant Acinetobacter baumannii Isolates from the Kingdom of Bahrain,” *Antibiotics*, vol. 12, no. 7, p. 1198, Jul. 2023, doi: 10.3390/antibiotics12071198.
[62] R. Vázquez-López *et al.*, “Acinetobacter baumannii Resistance: A Real Challenge for Clinicians,” *Antibiotics*, vol. 9, no. 4, p. 205, Apr. 2020, doi: 10.3390/antibiotics9040205.
[63] T. J. Silhavy, D. Kahne, and S. Walker, “The Bacterial Cell Envelope,” *Cold Spring Harb. Perspect. Biol.*, vol. 2, no. 5, p. a000414, May 2010, doi: 10.1101/cshperspect.a000414.
[64] J.-F. Collet, S.-H. Cho, B. I. Iorga, and C. V. Goemans, “How the assembly and protection of the bacterial cell envelope depend on cysteine residues,” *J. Biol. Chem.*, vol. 295, no. 34, pp. 11984–11994, Aug. 2020, doi: 10.1074/jbc.REV120.011201.
[65] T. J. Beveridge, “Structures of Gram-Negative Cell Walls and Their Derived Membrane Vesicles,” *J. Bacteriol.*, vol. 181, no. 16, pp. 4725–4733, Aug. 1999, doi: 10.1128/jb.181.16.4725-4733.1999.
[66] B. Bertani and N. Ruiz, “Function and biogenesis of lipopolysaccharides,” *EcoSal Plus*, vol. 8, no. 1, p. 10.1128/ecosalplus.ESP-0001–2018, Aug. 2018, doi: 10.1128/ecosalplus.ESP-0001-2018.
[67] J. W. Fairman, N. Noinaj, and S. K. Buchanan, “The structural biology of β-barrel membrane proteins: a summary of recent reports,” *Curr. Opin. Struct. Biol.*, vol. 21, no. 4, pp. 523–531, Aug. 2011, doi: 10.1016/j.sbi.2011.05.005.
[68] H. Tokuda, “Biogenesis of outer membranes in Gram-negative bacteria,” *Biosci. Biotechnol. Biochem.*, vol. 73, no. 3, pp. 465–473, Mar. 2009, doi: 10.1271/bbb.80778.
[69] C. Schwechheimer and M. J. Kuehn, “Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions,” *Nat. Rev. Microbiol.*, vol. 13, no. 10, pp. 605–619, Oct. 2015, doi: 10.1038/nrmicro3525.
[70] A. J. F. Egan, J. Errington, and W. Vollmer, “Regulation of peptidoglycan synthesis and remodelling,” *Nat. Rev. Microbiol.*, vol. 18, no. 8, Art. no. 8, Aug. 2020, doi: 10.1038/s41579-020-0366-3.
[71] A. Typas *et al.*, “Regulation of peptidoglycan synthesis by outer membrane proteins,” *Cell*, vol. 143, no. 7, pp. 1097–1109, Dec. 2010, doi: 10.1016/j.cell.2010.11.038.
[72] A. K. Sharma, S. Kumar, H. K., D. B. Dhakan, and V. K. Sharma, “Prediction of peptidoglycan hydrolases- a new class of antibacterial proteins,” *BMC Genomics*, vol. 17, p. 411, May 2016, doi: 10.1186/s12864-016-2753-8.
[73] J. W. Johnson, J. F. Fisher, and S. Mobashery, “Bacterial cell-wall recycling,” *Ann. N. Y. Acad. Sci.*, vol. 1277, no. 1, pp. 54–75, Jan. 2013, doi: 10.1111/j.1749-6632.2012.06813.x.
[74] D. A. Dik, D. R. Marous, J. F. Fisher, and S. Mobashery, “Lytic Transglycosylases: Concinnity in concision of the bacterial cell wall,” *Crit. Rev. Biochem. Mol. Biol.*, vol. 52, no. 5, pp. 503–542, Oct. 2017, doi: 10.1080/10409238.2017.1337705.
[75] M. Lee *et al.*, “Reactions of All E. coli Lytic Transglycosylases with Bacterial Cell Wall,” *J. Am. Chem. Soc.*, vol. 135, no. 9, pp. 3311–3314, Mar. 2013, doi: 10.1021/ja309036q.
[76] B. T. Cookson, A. N. Tyler, and W. E. Goldman, “Primary structure of the peptidoglycan-derived tracheal cytotoxin of Bordetella pertussis,” *Biochemistry*, vol. 28, no. 4, pp. 1744–1749, Feb. 1989, doi: 10.1021/bi00430a048.

[77] T. Uehara, K. Suefuji, N. Valbuena, B. Meehan, M. Donegan, and J. T. Park, "Recycling of the anhydro-N-acetylmuramic acid derived from cell wall murein involves a two-step conversion to N-acetylglucosamine-phosphate," *J. Bacteriol.*, vol. 187, no. 11, pp. 3643–3649, Jun. 2005, doi: 10.1128/JB.187.11.3643-3649.2005.

[78] D. L. Greenway and H. R. Perkins, "Turnover of the cell wall peptidoglycan during growth of Neisseria gonorrhoeae and Escherichia coli. Relative stability of newly synthesized material," *J. Gen. Microbiol.*, vol. 131, no. 2, pp. 253–263, Feb. 1985, doi: 10.1099/00221287-131-2-253.

[79] M. C. Gilmore and F. Cava, "Peptidoglycan recycling mediated by an ABC transporter in the plant pathogen Agrobacterium tumefaciens," *Nat. Commun.*, vol. 13, no. 1, Art. no. 1, Dec. 2022, doi: 10.1038/s41467-022-35607-5.

[80] S. López-Argüello, M. Montaner, A. Oliver, and B. Moya, "Molecular Basis of AmpC β-Lactamase Induction by Avibactam in Pseudomonas aeruginosa: PBP Occupancy, Live Cell Binding Dynamics and Impact on Resistant Clinical Isolates Harboring PDC-X Variants," *Int. J. Mol. Sci.*, vol. 22, no. 6, Art. no. 6, Jan. 2021, doi: 10.3390/ijms22063051.

[81] J. T. Park and T. Uehara, "How Bacteria Consume Their Own Exoskeletons (Turnover and Recycling of Cell Wall Peptidoglycan)," *Microbiol. Mol. Biol. Rev. MMBR*, vol. 72, no. 2, pp. 211–227, Jun. 2008, doi: 10.1128/MMBR.00027-07.

[82] M. I. Kazi, R. D. Schargel, and J. M. Boll, "Generating Transposon Insertion Libraries in Gram-Negative Bacteria for High-Throughput Sequencing," *J. Vis. Exp. JoVE*, no. 161, p. 10.3791/61612, Jul. 2020, doi: 10.3791/61612.

[83] A. K. Cain, L. Barquist, A. L. Goodman, I. T. Paulsen, J. Parkhill, and T. van Opijnen, "A decade of advances in transposon-insertion sequencing," *Nat. Rev. Genet.*, vol. 21, no. 9, Art. no. 9, Sep. 2020, doi: 10.1038/s41576-020-0244-x.

[84] J. D. D. Pitout, G. Peirano, M. M. Kock, K.-A. Strydom, and Y. Matsumura, "The Global Ascendency of OXA-48-Type Carbapenemases," *Clin. Microbiol. Rev.*, vol. 33, no. 1, p. 10.1128/cmr.00102-19, Nov. 2019, doi: 10.1128/cmr.00102-19.

[85] T. Naas *et al.*, "Beta-lactamase database (BLDB) - structure and function," *J. Enzyme Inhib. Med. Chem.*, vol. 32, no. 1, pp. 917–919, Dec. 2017, doi: 10.1080/14756366.2017.1344235.

[86] C. L. Tooke *et al.*, "β-Lactamases and β-Lactamase Inhibitors in the 21st Century," *J. Mol. Biol.*, vol. 431, no. 18, pp. 3472–3500, Aug. 2019, doi: 10.1016/j.jmb.2019.04.002.

[87] K. Bush and G. A. Jacoby, "Updated Functional Classification of β-Lactamases," *Antimicrob. Agents Chemother.*, vol. 54, no. 3, pp. 969–976, Mar. 2010, doi: 10.1128/AAC.01009-09.

[88] T. Palzkill, "Metallo-β-lactamase structure and function," *Ann. N. Y. Acad. Sci.*, vol. 1277, pp. 91–104, Jan. 2013, doi: 10.1111/j.1749-6632.2012.06796.x.

[89] D. J. Hong, I. K. Bae, I.-H. Jang, S. H. Jeong, H.-K. Kang, and K. Lee, "Epidemiology and Characteristics of Metallo-β-Lactamase-Producing Pseudomonas aeruginosa," *Infect. Chemother.*, vol. 47, no. 2, pp. 81–97, Jun. 2015, doi: 10.3947/ic.2015.47.2.81.

[90] Y. He, J. Lei, X. Pan, X. Huang, and Y. Zhao, "The hydrolytic water molecule of Class A β-lactamase relies on the acyl-enzyme intermediate ES* for proper coordination and catalysis," *Sci. Rep.*, vol. 10, no. 1, Art. no. 1, Jun. 2020, doi: 10.1038/s41598-020-66431-w.

[91] A. Steimle, I. B. Autenrieth, and J.-S. Frick, "Structure and function: Lipid A modifications in commensals and pathogens," *Int. J. Med. Microbiol.*, vol. 306, no. 5, pp. 290–301, Aug. 2016, doi: 10.1016/j.ijmm.2016.03.001.

[92] "Lipid A - an overview | ScienceDirect Topics." Accessed: Sep. 25, 2023. [Online]. Available: https://www.sciencedirect.com/topics/medicine-and-dentistry/lipid-a
[93] N. Kresge, R. D. Simoni, and R. L. Hill, "The Lipid A Assembly Pathway: The Work of Christian Raetz," *J. Biol. Chem.*, vol. 286, no. 29, pp. e6–e8, Jul. 2011, doi: 10.1074/jbc.O111.000247.
[94] C. R. H. Raetz, C. M. Reynolds, M. S. Trent, and R. E. Bishop, "LIPID A MODIFICATION SYSTEMS IN GRAM-NEGATIVE BACTERIA," *Annu. Rev. Biochem.*, vol. 76, pp. 295–329, 2007, doi: 10.1146/annurev.biochem.76.010307.145803.
[95] P. Zhang, Z. Ye, C. Ye, H. Zou, Z. Gao, and J. Pan, "OmpW is positively regulated by iron via Fur, and negatively regulated by SoxS contribution to oxidative stress resistance in Escherichia coli," *Microb. Pathog.*, vol. 138, p. 103808, Jan. 2020, doi: 10.1016/j.micpath.2019.103808.
[96] E. Sugawara and H. Nikaido, "OmpA Is the Principal Nonspecific Slow Porin of Acinetobacter baumannii," *J. Bacteriol.*, vol. 194, no. 15, pp. 4089–4096, Aug. 2012, doi: 10.1128/JB.00435-12.
[97] V. Tiku *et al.*, "Outer membrane vesicles containing OmpA induce mitochondrial fragmentation to promote pathogenesis of Acinetobacter baumannii," *Sci. Rep.*, vol. 11, no. 1, Art. no. 1, Jan. 2021, doi: 10.1038/s41598-020-79966-9.
[98] P. J. F. Henderson, C. Maher, L. D. H. Elbourne, B. A. Eijkelkamp, I. T. Paulsen, and K. A. Hassan, "Physiological Functions of Bacterial 'Multidrug' Efflux Pumps," *Chem. Rev.*, vol. 121, no. 9, pp. 5417–5478, May 2021, doi: 10.1021/acs.chemrev.0c01226.
[99] J. Anes, M. P. McCusker, S. Fanning, and M. Martins, "The ins and outs of RND efflux pumps in Escherichia coli," *Front. Microbiol.*, vol. 6, p. 587, Jun. 2015, doi: 10.3389/fmicb.2015.00587.
[100] P. Verma, M. Tiwari, and V. Tiwari, "Efflux pumps in multidrug-resistant Acinetobacter baumannii: Current status and challenges in the discovery of efflux pumps inhibitors," *Microb. Pathog.*, vol. 152, p. 104766, Mar. 2021, doi: 10.1016/j.micpath.2021.104766.
[101] E. Sugawara and H. Nikaido, "Properties of AdeABC and AdeIJK Efflux Systems of Acinetobacter baumannii Compared with Those of the AcrAB-TolC System of Escherichia coli," *Antimicrob. Agents Chemother.*, vol. 58, no. 12, pp. 7250–7257, Dec. 2014, doi: 10.1128/AAC.03728-14.
[102] K. Nishino, S. Yamasaki, R. Nakashima, M. Zwama, and M. Hayashi-Nishino, "Function and Inhibitory Mechanisms of Multidrug Efflux Pumps," *Front. Microbiol.*, vol. 12, 2021, Accessed: Sep. 30, 2023. [Online]. Available: https://www.frontiersin.org/articles/10.3389/fmicb.2021.737288
[103] E. R. Rojas *et al.*, "The outer membrane is an essential load-bearing element in Gram-negative bacteria," *Nature*, vol. 559, no. 7715, pp. 617–621, 2018, doi: 10.1038/s41586-018-0344-3.
[104] N. Morè *et al.*, "Peptidoglycan Remodeling Enables Escherichia coli To Survive Severe Outer Membrane Assembly Defect," *mBio*, vol. 10, no. 1, 05 2019, doi: 10.1128/mBio.02729-18.
[105] I. Levin-Reisman, I. Ronin, O. Gefen, I. Braniss, N. Shoresh, and N. Q. Balaban, "Antibiotic tolerance facilitates the evolution of resistance," *Science*, vol. 355, no. 6327, pp. 826–830, Feb. 2017, doi: 10.1126/science.aaj2191.

[106] I. Levin-Reisman, A. Brauner, I. Ronin, and N. Q. Balaban, “Epistasis between antibiotic tolerance, persistence, and resistance mutations,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 116, no. 29, pp. 14734–14739, Jul. 2019, doi: 10.1073/pnas.1906169116.
[107] J. Liu, O. Gefen, I. Ronin, M. Bar-Meir, and N. Q. Balaban, “Effect of tolerance on the evolution of antibiotic resistance under drug combinations,” *Science*, vol. 367, no. 6474, pp. 200–204, Jan. 2020, doi: 10.1126/science.aay3041.
[108] A. Brauner, O. Fridman, O. Gefen, and N. Q. Balaban, “Distinguishing between resistance, tolerance and persistence to antibiotic treatment,” *Nat. Rev. Microbiol.*, vol. 14, no. 5, pp. 320–330, 2016, doi: 10.1038/nrmicro.2016.34.
[109] T. Dörr, “Understanding tolerance to cell wall-active antibiotics,” *Ann. N. Y. Acad. Sci.*, Dec. 2020, doi: 10.1111/nyas.14541.
[110] L. F. Westblade, J. Errington, and T. Dörr, “Antibiotic tolerance,” *PLoS Pathog.*, vol. 16, no. 10, p. e1008892, Oct. 2020, doi: 10.1371/journal.ppat.1008892.
[111] K. M. Papp-Wallace, A. Endimiani, M. A. Taracila, and R. A. Bonomo, “Carbapenems: past, present, and future,” *Antimicrob. Agents Chemother.*, vol. 55, no. 11, pp. 4943–4960, Nov. 2011, doi: 10.1128/AAC.00296-11.
[112] M. Bassetti, L. Nicolini, S. Esposito, E. Righi, and C. Viscoli, “Current status of newer carbapenems,” *Curr. Med. Chem.*, vol. 16, no. 5, pp. 564–575, 2009, doi: 10.2174/092986709787458498.
[113] J. A. Torres, M. V. Villegas, and J. P. Quinn, “Current concepts in antibiotic-resistant gram-negative bacteria,” *Expert Rev. Anti Infect. Ther.*, vol. 5, no. 5, pp. 833–843, Oct. 2007, doi: 10.1586/14787210.5.5.833.
[114] D. L. Paterson and R. A. Bonomo, “Extended-spectrum beta-lactamases: a clinical update,” *Clin. Microbiol. Rev.*, vol. 18, no. 4, pp. 657–686, Oct. 2005, doi: 10.1128/CMR.18.4.657-686.2005.
[115] G. M. Eliopoulos, L. L. Maragakis, and T. M. Perl, “Acinetobacter baumannii: Epidemiology, Antimicrobial Resistance, and Treatment Options,” *Clin. Infect. Dis.*, vol. 46, no. 8, pp. 1254–1263, Apr. 2008, doi: 10.1086/529198.
[116] J. J. Rahal, “Antimicrobial resistance among and therapeutic options against gram-negative pathogens,” *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.*, vol. 49 Suppl 1, pp. S4–S10, Aug. 2009, doi: 10.1086/599810.
[117] CDC, “Antibiotic Resistance Threats in the United States, 2019,” p. 148, 2019.
[118] “WHO | Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics,” WHO. Accessed: Jan. 01, 2018. [Online]. Available: http://www.who.int/medicines/publications/global-priority-list-antibiotic-resistant-bacteria/en/
[119] T. Dörr, B. M. Davis, and M. K. Waldor, “Endopeptidase-mediated beta lactam tolerance,” *PLoS Pathog.*, vol. 11, no. 4, p. e1004850, Apr. 2015, doi: 10.1371/journal.ppat.1004850.
[120] A. I. Weaver *et al.*, “Genetic Determinants of Penicillin Tolerance in Vibrio cholerae,” *Antimicrob. Agents Chemother.*, vol. 62, no. 10, 2018, doi: 10.1128/AAC.01326-18.
[121] L. G. Monahan, L. Turnbull, S. R. Osvath, D. Birch, I. G. Charles, and C. B. Whitchurch, “Rapid conversion of Pseudomonas aeruginosa to a spherical cell morphotype facilitates tolerance to carbapenems and penicillins but increases susceptibility to antimicrobial peptides,” *Antimicrob. Agents Chemother.*, vol. 58, no. 4, pp. 1956–1962, 2014, doi: 10.1128/AAC.01901-13.

[122] M. I. Kazi, R. D. Schargel, and J. M. Boll, “Generating Transposon Insertion Libraries in Gram-Negative Bacteria for High-Throughput Sequencing,” *J. Vis. Exp. JoVE*, no. 161, 07 2020, doi: 10.3791/61612.

[123] A. T. Tucker *et al.*, “Discovery of Next-Generation Antimicrobials through Bacterial Self-Screening of Surface-Displayed Peptide Libraries,” *Cell*, vol. 172, no. 3, pp. 618-628.e13, Jan. 2018, doi: 10.1016/j.cell.2017.12.009.

[124] J. M. Boll *et al.*, “Reinforcing Lipid A Acylation on the Cell Surface of Acinetobacter baumannii Promotes Cationic Antimicrobial Peptide Resistance and Desiccation Survival,” *mBio*, vol. 6, no. 3, pp. e00478-00415, May 2015, doi: 10.1128/mBio.00478-15.

[125] T. Cross *et al.*, “Spheroplast-Mediated Carbapenem Tolerance in Gram-Negative Pathogens,” *Antimicrob. Agents Chemother.*, vol. 63, no. 9, pp. e00756-19, /aac/63/9/AAC.00756-19.atom, Jul. 2019, doi: 10.1128/AAC.00756-19.

[126] C. D. Fage, D. B. Brown, J. M. Boll, A. T. Keatinge-Clay, and M. S. Trent, “Crystallographic study of the phosphoethanolamine transferase EptC required for polymyxin resistance and motility in *Campylobacter jejuni*,” *Acta Crystallogr. D Biol. Crystallogr.*, vol. 70, no. 10, pp. 2730–2739, Oct. 2014, doi: 10.1107/S1399004714017623.

[127] K. N. Kang *et al.*, “Septal Class A Penicillin-Binding Protein Activity and LD -Transpeptidases Mediate Selection of Colistin-Resistant Lipooligosaccharide-Deficient Acinetobacter baumannii,” *mBio*, vol. 12, no. 1, Feb. 2021, doi: 10.1128/mBio.02185-20.

[128] C. L. Marolda, P. Lahiry, E. Vinés, S. Saldías, and M. A. Valvano, “Micromethods for the Characterization of Lipid A-Core and O-Antigen Lipopolysaccharide,” in *Glycobiology Protocols*, vol. 347, New Jersey: Humana Press, 2006, pp. 237–252. doi: 10.1385/1-59745-167-3:237.

[129] K. N. Kang *et al.*, “Colistin heteroresistance in *Enterobacter cloacae* is regulated by PhoPQ-dependent 4-amino-4-deoxy- L -arabinose addition to lipid A,” *Mol. Microbiol.*, vol. 111, no. 6, pp. 1604–1616, Jun. 2019, doi: 10.1111/mmi.14240.

[130] T. Cross *et al.*, “Spheroplast-Mediated Carbapenem Tolerance in Gram-Negative Pathogens,” *Antimicrob. Agents Chemother.*, vol. 63, no. 9, 2019, doi: 10.1128/AAC.00756-19.

[131] A. N. Murtha *et al.*, “High-level carbapenem tolerance requires antibiotic-induced outer membrane modifications,” *PLoS Pathog.*, vol. 18, no. 2, p. e1010307, Feb. 2022, doi: 10.1371/journal.ppat.1010307.

[132] G. I. McLeod and M. P. Spector, “Starvation- and Stationary-phase-induced resistance to the antimicrobial peptide polymyxin B in Salmonella typhimurium is RpoS (sigma(S)) independent and occurs through both phoP-dependent and -independent pathways,” *J. Bacteriol.*, vol. 178, no. 13, pp. 3683–3688, Jul. 1996, doi: 10.1128/jb.178.13.3683-3688.1996.

[133] E. Tuomanen, R. Cozens, W. Tosch, O. Zak, and A. Tomasz, “The rate of killing of Escherichia coli by beta-lactam antibiotics is strictly proportional to the rate of bacterial growth,” *J. Gen. Microbiol.*, vol. 132, no. 5, pp. 1297–1304, May 1986, doi: 10.1099/00221287-132-5-1297.

[134] A. J. Lee, S. Wang, H. R. Meredith, B. Zhuang, Z. Dai, and L. You, “Robust, linear correlations between growth rates and β-lactam-mediated lysis rates,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 115, no. 16, pp. 4069–4074, Apr. 2018, doi: 10.1073/pnas.1719504115.

[135] E. Kuru *et al.*, "Fluorescent D-amino-acids reveal bi-cellular cell wall modifications important for Bdellovibrio bacteriovorus predation," *Nat. Microbiol.*, vol. 2, no. 12, pp. 1648–1657, Dec. 2017, doi: 10.1038/s41564-017-0029-y.

[136] E. Kuru *et al.*, "Mechanisms of incorporation for D-amino acid probes that target peptidoglycan biosynthesis," *ACS Chem. Biol.*, Nov. 2019, doi: 10.1021/acschembio.9b00664.

[137] C. Baranowski *et al.*, "Maturing Mycobacterium smegmatis peptidoglycan requires non-canonical crosslinks to maintain shape," *eLife*, vol. 7, 16 2018, doi: 10.7554/eLife.37516.

[138] A. García-Heredia *et al.*, "Peptidoglycan precursor synthesis along the sidewall of pole-growing mycobacteria," *eLife*, vol. 7, 10 2018, doi: 10.7554/eLife.37243.

[139] M.-F. Lin, Y.-Y. Lin, C.-C. Tu, and C.-Y. Lan, "Distribution of different efflux pump genes in clinical isolates of multidrug-resistant Acinetobacter baumannii and their correlation with antimicrobial resistance," *J. Microbiol. Immunol. Infect. Wei Mian Yu Gan Ran Za Zhi*, vol. 50, no. 2, pp. 224–231, Apr. 2017, doi: 10.1016/j.jmii.2015.04.004.

[140] E.-J. Yoon, P. Courvalin, and C. Grillot-Courvalin, "RND-type efflux pumps in multidrug-resistant clinical isolates of Acinetobacter baumannii: major role for AdeABC overexpression and AdeRS mutations," *Antimicrob. Agents Chemother.*, vol. 57, no. 7, pp. 2989–2995, Jul. 2013, doi: 10.1128/AAC.02556-12.

[141] G. Rajamohan, V. B. Srinivasan, and W. A. Gebreyes, "Novel role of Acinetobacter baumannii RND efflux transporters in mediating decreased susceptibility to biocides," *J. Antimicrob. Chemother.*, vol. 65, no. 2, pp. 228–232, Feb. 2010, doi: 10.1093/jac/dkp427.

[142] L. Damier-Piolle, S. Magnet, S. Brémont, T. Lambert, and P. Courvalin, "AdeIJK, a resistance-nodulation-cell division pump effluxing multiple antibiotics in Acinetobacter baumannii," *Antimicrob. Agents Chemother.*, vol. 52, no. 2, pp. 557–562, Feb. 2008, doi: 10.1128/AAC.00732-07.

[143] V. Kornelsen and A. Kumar, "Update on Multidrug Resistance Efflux Pumps in Acinetobacter spp," *Antimicrob. Agents Chemother.*, vol. 65, no. 7, p. e0051421, Jun. 2021, doi: 10.1128/AAC.00514-21.

[144] J.-M. Pagès, C. E. James, and M. Winterhalter, "The porin and the permeating antibiotic: a selective diffusion barrier in Gram-negative bacteria," *Nat. Rev. Microbiol.*, vol. 6, no. 12, pp. 893–903, Dec. 2008, doi: 10.1038/nrmicro1994.

[145] M. A. Mussi, V. M. Relling, A. S. Limansky, and A. M. Viale, "CarO, an Acinetobacter baumannii outer membrane protein involved in carbapenem resistance, is essential for L-ornithine uptake," *FEBS Lett.*, vol. 581, no. 29, pp. 5573–5578, Dec. 2007, doi: 10.1016/j.febslet.2007.10.063.

[146] M. A. Mussi, A. S. Limansky, and A. M. Viale, "Acquisition of resistance to carbapenems in multidrug-resistant clinical strains of Acinetobacter baumannii: natural insertional inactivation of a gene encoding a member of a novel family of beta-barrel outer membrane proteins," *Antimicrob. Agents Chemother.*, vol. 49, no. 4, pp. 1432–1440, Apr. 2005, doi: 10.1128/AAC.49.4.1432-1440.2005.

[147] M. Dupont, J.-M. Pagès, D. Lafitte, A. Siroy, and C. Bollet, "Identification of an OprD homologue in Acinetobacter baumannii," *J. Proteome Res.*, vol. 4, no. 6, pp. 2386–2390, Dec. 2005, doi: 10.1021/pr050143q.

[148] M. Catel-Ferreira *et al.*, "The outer membrane porin OmpW of Acinetobacter baumannii is involved in iron uptake and colistin binding," *FEBS Lett.*, vol. 590, no. 2, pp. 224–231, Jan. 2016, doi: 10.1002/1873-3468.12050.

[149] J.-H. Shin *et al.*, "A multifaceted cellular damage repair and prevention pathway promotes high-level tolerance to β-lactam antibiotics," *EMBO Rep.*, vol. 22, no. 2, p. e51790, Feb. 2021, doi: 10.15252/embr.202051790.
[150] T. Dörr, L. Alvarez, F. Delgado, B. M. Davis, F. Cava, and M. K. Waldor, "A cell wall damage response mediated by a sensor kinase/response regulator pair enables beta-lactam tolerance," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 113, no. 2, pp. 404–409, Jan. 2016, doi: 10.1073/pnas.1520333113.
[151] J. M. Boll *et al.*, "A penicillin-binding protein inhibits selection of colistin-resistant, lipooligosaccharide-deficient Acinetobacter baumannii," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 113, no. 41, pp. E6228–E6237, 11 2016, doi: 10.1073/pnas.1611594113.
[152] R. Henry *et al.*, "Colistin-resistant, lipopolysaccharide-deficient Acinetobacter baumannii responds to lipopolysaccharide loss through increased expression of genes involved in the synthesis and transport of lipoproteins, phospholipids, and poly-β-1,6-N-acetylglucosamine," *Antimicrob. Agents Chemother.*, vol. 56, no. 1, pp. 59–69, Jan. 2012, doi: 10.1128/AAC.05191-11.
[153] R. Henry *et al.*, "The transcriptomic response of Acinetobacter baumannii to colistin and doripenem alone and in combination in an in vitro pharmacokinetics/pharmacodynamics model," *J. Antimicrob. Chemother.*, vol. 70, no. 5, pp. 1303–1313, May 2015, doi: 10.1093/jac/dku536.
[154] M. Mathelié-Guinlet, A. T. Asmar, J.-F. Collet, and Y. F. Dufrêne, "Lipoprotein Lpp regulates the mechanical properties of the E. coli cell envelope," *Nat. Commun.*, vol. 11, no. 1, p. 1789, Apr. 2020, doi: 10.1038/s41467-020-15489-1.
[155] E. M. Scheurwater and A. J. Clarke, "The C-terminal domain of Escherichia coli YfhD functions as a lytic transglycosylase," *J. Biol. Chem.*, vol. 283, no. 13, pp. 8363–8373, Mar. 2008, doi: 10.1074/jbc.M710135200.
[156] T. Uehara and J. T. Park, "Peptidoglycan Recycling," *EcoSal Plus*, vol. 3, no. 1, Sep. 2008, doi: 10.1128/ecosalplus.4.7.1.5.
[157] O. Irazoki, S. B. Hernandez, and F. Cava, "Peptidoglycan Muropeptides: Release, Perception, and Functions as Signaling Molecules," *Front. Microbiol.*, vol. 10, p. 500, 2019, doi: 10.3389/fmicb.2019.00500.
[158] K. N. Kang *et al.*, "Septal Class A Penicillin-Binding Protein Activity and ld-Transpeptidases Mediate Selection of Colistin-Resistant Lipooligosaccharide-Deficient Acinetobacter baumannii," *mBio*, vol. 12, no. 1, pp. e02185-20, Jan. 2021, doi: 10.1128/mBio.02185-20.
[159] M. I. Kazi, R. D. Schargel, and J. M. Boll, "Generating Transposon Insertion Libraries in Gram-Negative Bacteria for High-Throughput Sequencing," *JoVE J. Vis. Exp.*, no. 161, p. e61612, Jul. 2020, doi: 10.3791/61612.
[160] E. Geisinger *et al.*, "Antibiotic susceptibility signatures identify potential antimicrobial targets in the Acinetobacter baumannii cell envelope," *Nat. Commun.*, vol. 11, no. 1, p. 4522, 09 2020, doi: 10.1038/s41467-020-18301-2.
[161] J. S. Park *et al.*, "Mechanism of anchoring of OmpA protein to the cell wall peptidoglycan of the gram-negative bacterial outer membrane," *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.*, vol. 26, no. 1, pp. 219–228, Jan. 2012, doi: 10.1096/fj.11-188425.
[162] A. Gribun, Y. Nitzan, I. Pechatnikov, G. Hershkovits, and D. J. Katcoff, "Molecular and structural characterization of the HMP-AB gene encoding a pore-forming protein from a

clinical isolate of Acinetobacter baumannii," *Curr. Microbiol.*, vol. 47, no. 5, pp. 434–443, Nov. 2003, doi: 10.1007/s00284-003-4050-4.

[163] Y. Smani, M. J. McConnell, and J. Pachón, "Role of fibronectin in the adhesion of Acinetobacter baumannii to host cells," *PloS One*, vol. 7, no. 4, p. e33073, 2012, doi: 10.1371/journal.pone.0033073.

[164] Y.-K. Tsai, C.-H. Liou, J.-C. Lin, C.-P. Fung, F.-Y. Chang, and L. K. Siu, "Effects of different resistance mechanisms on antimicrobial resistance in Acinetobacter baumannii: a strategic system for screening and activity testing of new antibiotics," *Int. J. Antimicrob. Agents*, vol. 55, no. 4, p. 105918, Apr. 2020, doi: 10.1016/j.ijantimicag.2020.105918.

[165] D. C. Moon *et al.*, "Acinetobacter baumannii outer membrane protein A modulates the biogenesis of outer membrane vesicles," *J. Microbiol. Seoul Korea*, vol. 50, no. 1, pp. 155–160, Feb. 2012, doi: 10.1007/s12275-012-1589-4.

[166] J. M. Boll *et al.*, "Reinforcing Lipid A Acylation on the Cell Surface of Acinetobacter baumannii Promotes Cationic Antimicrobial Peptide Resistance and Desiccation Survival," *mBio*, vol. 6, no. 3, pp. e00478-00415, 2015, doi: 10.1128/mBio.00478-15.

[167] J. E. Horne, D. J. Brockwell, and S. E. Radford, "Role of the lipid bilayer in outer membrane protein folding in Gram-negative bacteria," *J. Biol. Chem.*, vol. 295, no. 30, pp. 10340–10367, Jul. 2020, doi: 10.1074/jbc.REV120.011473.

[168] K. M. Storek *et al.*, "Monoclonal antibody targeting the β-barrel assembly machine of Escherichia coli is bactericidal," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 115, no. 14, pp. 3692–3697, Apr. 2018, doi: 10.1073/pnas.1800043115.

[169] N.-H. Le *et al.*, "Peptidoglycan editing provides immunity to Acinetobacter baumannii during bacterial warfare," *Sci. Adv.*, vol. 6, no. 30, p. eabb5614, Jul. 2020, doi: 10.1126/sciadv.abb5614.

[170] D. H. Edwards and W. D. Donachie, "Construction of a Triple Deletion of Penicillin-Binding Proteins 4, 5, and 6 in Escherichia coli," in *Bacterial Growth and Lysis: Metabolism and Structure of the Bacterial Sacculus*, M. A. de Pedro, J.-V. Höltje, and W. Löffelhardt, Eds., in Federation of European Microbiological Societies Symposium Series. , Boston, MA: Springer US, 1993, pp. 369–374. doi: 10.1007/978-1-4757-9359-8_44.

[171] L. Potluri *et al.*, "Septal and lateral wall localization of PBP5, the major D,D-carboxypeptidase of Escherichia coli, requires substrate recognition and membrane attachment," *Mol. Microbiol.*, vol. 77, no. 2, pp. 300–323, Jul. 2010, doi: 10.1111/j.1365-2958.2010.07205.x.

[172] U. Kohlrausch and J. V. Höltje, "Analysis of murein and murein precursors during antibiotic-induced lysis of Escherichia coli," *J. Bacteriol.*, vol. 173, no. 11, pp. 3425–3431, Jun. 1991, doi: 10.1128/jb.173.11.3425-3431.1991.

[173] M. F. Templin, A. Ursinus, and J. V. Höltje, "A defect in cell wall recycling triggers autolysis during the stationary growth phase of Escherichia coli," *EMBO J.*, vol. 18, no. 15, pp. 4108–4117, Aug. 1999, doi: 10.1093/emboj/18.15.4108.

[174] Q. Cheng and J. T. Park, "Substrate specificity of the AmpG permease required for recycling of cell wall anhydro-muropeptides," *J. Bacteriol.*, vol. 184, no. 23, pp. 6434–6436, Dec. 2002, doi: 10.1128/JB.184.23.6434-6436.2002.

[175] W. Vötsch and M. F. Templin, "Characterization of a beta -N-acetylglucosaminidase of Escherichia coli and elucidation of its role in muropeptide recycling and beta -lactamase induction," *J. Biol. Chem.*, vol. 275, no. 50, pp. 39032–39038, Dec. 2000, doi: 10.1074/jbc.M004797200.

[176] J. V. Höltje, U. Kopp, A. Ursinus, and B. Wiedemann, "The negative regulator of beta-lactamase induction AmpD is a N-acetyl-anhydromuramyl-L-alanine amidase," *FEMS Microbiol. Lett.*, vol. 122, no. 1–2, pp. 159–164, Sep. 1994, doi: 10.1111/j.1574-6968.1994.tb07159.x.

[177] C. Jacobs *et al.*, "AmpD, essential for both beta-lactamase regulation and cell wall recycling, is a novel cytosolic N-acetylmuramyl-L-alanine amidase," *Mol. Microbiol.*, vol. 15, no. 3, pp. 553–559, Feb. 1995, doi: 10.1111/j.1365-2958.1995.tb02268.x.

[178] D. Mengin-Lecreulx, J. van Heijenoort, and J. T. Park, "Identification of the mpl gene encoding UDP-N-acetylmuramate: L-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase in Escherichia coli and its role in recycling of cell wall peptidoglycan," *J. Bacteriol.*, vol. 178, no. 18, pp. 5347–5352, Sep. 1996, doi: 10.1128/jb.178.18.5347-5352.1996.

[179] R. Bahadur, P. K. Chodisetti, and M. Reddy, "Cleavage of Braun's lipoprotein Lpp from the bacterial peptidoglycan by a paralog of l,d-transpeptidases, LdtF," *Proc. Natl. Acad. Sci.*, vol. 118, no. 19, May 2021, doi: 10.1073/pnas.2101989118.

[180] M. J. Powers and M. S. Trent, "Phospholipid retention in the absence of asymmetry strengthens the outer membrane permeability barrier to last-resort antibiotics," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 115, no. 36, pp. E8518–E8527, 04 2018, doi: 10.1073/pnas.1806714115.

[181] S. Magnet *et al.*, "Identification of the L,D-transpeptidases responsible for attachment of the Braun lipoprotein to Escherichia coli peptidoglycan," *J. Bacteriol.*, vol. 189, no. 10, pp. 3927–3931, May 2007, doi: 10.1128/JB.00084-07.

[182] Y. Dai, V. Pinedo, A. Y. Tang, F. Cava, and E. Geisinger, "A new class of cell wall-recycling L,D-carboxypeptidase determines β-lactam susceptibility and morphogenesis in Acinetobacter baumannii," Sep. 2021. doi: 10.1101/2021.09.22.461454.

[183] B. W. Simpson, M. Nieckarz, V. Pinedo, A. B. McLean, F. Cava, and M. S. Trent, "Acinetobacter baumannii Can Survive with an Outer Membrane Lacking Lipooligosaccharide Due to Structural Support from Elongasome Peptidoglycan Synthesis," *mBio*, vol. 12, no. 6, p. e0309921, Dec. 2021, doi: 10.1128/mBio.03099-21.

[184] U. Schroeder, B. Henrich, J. Fink, and R. Plapp, "Peptidase D of Escherichia coli K-12, a metallopeptidase of low substrate specificity," *FEMS Microbiol. Lett.*, vol. 123, no. 1–2, pp. 153–159, Oct. 1994, doi: 10.1111/j.1574-6968.1994.tb07215.x.

[185] D. M. Schmidt, B. K. Hubbard, and J. A. Gerlt, "Evolution of enzymatic activities in the enolase superfamily: functional assignment of unknown proteins in Bacillus subtilis and Escherichia coli as L-Ala-D/L-Glu epimerases," *Biochemistry*, vol. 40, no. 51, pp. 15707–15715, Dec. 2001, doi: 10.1021/bi011640x.

[186] D. Das *et al.*, "Structure and function of the first full-length murein peptide ligase (Mpl) cell wall recycling protein," *PloS One*, vol. 6, no. 3, p. e17624, Mar. 2011, doi: 10.1371/journal.pone.0017624.

[187] R. J. White and C. A. Pasternak, "The purification and properties of N-acetylglucosamine 6-phosphate deacetylase from Escherichia coli," *Biochem. J.*, vol. 105, no. 1, pp. 121–125, Oct. 1967, doi: 10.1042/bj1050121.

[188] T. Uehara, K. Suefuji, N. Valbuena, B. Meehan, M. Donegan, and J. T. Park, "Recycling of the anhydro-N-acetylmuramic acid derived from cell wall murein involves a two-step conversion to N-acetylglucosamine-phosphate," *J. Bacteriol.*, vol. 187, no. 11, pp. 3643–3649, Jun. 2005, doi: 10.1128/JB.187.11.3643-3649.2005.

[189] T. Uehara, K. Suefuji, T. Jaeger, C. Mayer, and J. T. Park, “MurQ Etherase is required by Escherichia coli in order to metabolize anhydro-N-acetylmuramic acid obtained either from the environment or from its own cell wall,” *J. Bacteriol.*, vol. 188, no. 4, pp. 1660–1662, Feb. 2006, doi: 10.1128/JB.188.4.1660-1662.2006.

[190] M. C. F. van Teeseling, M. A. de Pedro, and F. Cava, “Determinants of Bacterial Morphology: From Fundamentals to Possibilities for Antimicrobial Targeting,” *Front. Microbiol.*, vol. 8, 2017, Accessed: Oct. 25, 2023. [Online]. Available: https://www.frontiersin.org/articles/10.3389/fmicb.2017.01264

[191] E. M. Fivenson *et al.*, “A role for the Gram-negative outer membrane in bacterial shape determination,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 120, no. 35, p. e2301987120, doi: 10.1073/pnas.2301987120.

[192] M. C. F. van Teeseling, “Elongation at Midcell in Preparation of Cell Division Requires FtsZ, but Not MreB nor PBP2 in Caulobacter crescentus,” *Front. Microbiol.*, vol. 12, p. 732031, Aug. 2021, doi: 10.3389/fmicb.2021.732031.

[193] A. Tajuelo, M. C. Terrón, M. López-Siles, and M. J. McConnell, “Role of peptidoglycan recycling enzymes AmpD and AnmK in Acinetobacter baumannii virulence features,” *Front. Cell. Infect. Microbiol.*, vol. 12, 2023, Accessed: Oct. 26, 2023. [Online]. Available: https://www.frontiersin.org/articles/10.3389/fcimb.2022.1064053

[194] F. Ahmed, M. T. Mahmud, S. Naher, M. J. Rana, R. Ara, and K. Saif-Ur-Rahman, “Effectiveness of Colistin in carbapenem resistant Acinetobacter baumannii – A systematic review,” *Health Sci. Rev.*, vol. 8, p. 100113, Sep. 2023, doi: 10.1016/j.hsr.2023.100113.

[195] K. A. Glen and I. L. Lamont, “β-lactam Resistance in Pseudomonas aeruginosa: Current Status, Future Prospects,” *Pathogens*, vol. 10, no. 12, p. 1638, Dec. 2021, doi: 10.3390/pathogens10121638.

[196] J. M. Colquhoun *et al.*, “OXA-23 β-Lactamase Overexpression in Acinetobacter baumannii Drives Physiological Changes Resulting in New Genetic Vulnerabilities,” *mBio*, vol. 12, no. 6, pp. e03137-21, doi: 10.1128/mBio.03137-21.

[197] K. N. Kang *et al.*, “Septal Class A Penicillin-Binding Protein Activity and ld-Transpeptidases Mediate Selection of Colistin-Resistant Lipooligosaccharide-Deficient Acinetobacter baumannii,” *mBio*, vol. 12, no. 1, pp. e02185-20, Jan. 2021, doi: 10.1128/mBio.02185-20.

[198] J. Schindelin *et al.*, “Fiji - an Open Source platform for biological image analysis,” *Nat. Methods*, vol. 9, no. 7, p. 10.1038/nmeth.2019, Jun. 2012, doi: 10.1038/nmeth.2019.

[199] A. Ducret, E. M. Quardokus, and Y. V. Brun, “MicrobeJ, a tool for high throughput bacterial cell detection and quantitative analysis,” *Nat. Microbiol.*, vol. 1, no. 7, p. 16077, Jun. 2016, doi: 10.1038/nmicrobiol.2016.77.

[200] A. T. Tucker *et al.*, “Defining Gene-Phenotype Relationships in Acinetobacter baumannii through One-Step Chromosomal Gene Inactivation,” *mBio*, vol. 5, no. 4, p. 10.1128/mbio.01313-14, Aug. 2014, doi: 10.1128/mbio.01313-14.

[201] P. D. Lister, D. J. Wolter, and N. D. Hanson, “Antibacterial-Resistant Pseudomonas aeruginosa: Clinical Impact and Complex Regulation of Chromosomally Encoded Resistance Mechanisms,” *Clin. Microbiol. Rev.*, vol. 22, no. 4, pp. 582–610, Oct. 2009, doi: 10.1128/CMR.00040-09.

[202] C. Jacobs, L. J. Huang, E. Bartowsky, S. Normark, and J. T. Park, “Bacterial cell wall recycling provides cytosolic muropeptides as effectors for beta-lactamase induction,”

EMBO J., vol. 13, no. 19, pp. 4684–4694, Oct. 1994, doi: 10.1002/j.1460-2075.1994.tb06792.x.

[203] H. Dietz and B. Wiedemann, "The role of N-actylglucosaminyl-1,6 anhydro N-acetylmuramyl-L-alanyl-D-glutamyl-meso-diaminopimelic acid-D-alanine for the induction of beta-lactamase in Enterobacter cloacae," *Zentralblatt Bakteriol. Int. J. Med. Microbiol.*, vol. 284, no. 2–3, pp. 207–217, Jul. 1996, doi: 10.1016/s0934-8840(96)80096-x.

[204] B. Wiedemann, H. Dietz, and D. Pfeifle, "Induction of beta-lactamase in Enterobacter cloacae," *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.*, vol. 27 Suppl 1, pp. S42-47, Aug. 1998, doi: 10.1086/514921.

[205] M. H. Al-Agamy, N. G. Khalaf, M. M. Tawfick, A. M. Shibl, and A. E. Kholy, "Molecular characterization of carbapenem-insensitive Acinetobacter baumannii in Egypt," *Int. J. Infect. Dis.*, vol. 22, pp. 49–54, May 2014, doi: 10.1016/j.ijid.2013.12.004.

[206] B. Périchon *et al.*, "Identification of 50 Class D β-Lactamases and 65 Acinetobacter-Derived Cephalosporinases in Acinetobacter spp.," *Antimicrob. Agents Chemother.*, vol. 58, no. 2, pp. 936–949, Feb. 2014, doi: 10.1128/AAC.01261-13.

[207] E. Zander, A. Nemec, H. Seifert, and P. G. Higgins, "Association between β-Lactamase-Encoding blaOXA-51 Variants and DiversiLab Rep-PCR-Based Typing of Acinetobacter baumannii Isolates," *J. Clin. Microbiol.*, vol. 50, no. 6, pp. 1900–1904, Jun. 2012, doi: 10.1128/JCM.06462-11.

[208] J. V. Höltje, U. Kopp, A. Ursinus, and B. Wiedemann, "The negative regulator of beta-lactamase induction AmpD is a N-acetyl-anhydromuramyl-L-alanine amidase," *FEMS Microbiol. Lett.*, vol. 122, no. 1–2, pp. 159–164, Sep. 1994, doi: 10.1111/j.1574-6968.1994.tb07159.x.

[209] B. A. Bachert and J. A. Bozue, "Peptidoglycan enzymes of Francisella: Roles in cell morphology and pathogenesis, and potential as therapeutic targets," *Front. Microbiol.*, vol. 13, 2023, Accessed: Nov. 20, 2023. [Online]. Available: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1099312

[210] Y. Dai, V. Pinedo, A. Y. Tang, F. Cava, and E. Geisinger, "A New Class of Cell Wall-Recycling l,d-Carboxypeptidase Determines β-Lactam Susceptibility and Morphogenesis in Acinetobacter baumannii," *mBio*, vol. 12, no. 6, pp. e02786-21, Dec. 2021, doi: 10.1128/mBio.02786-21.

[211] K. A. Stubbs *et al.*, "The Development of Selective Inhibitors of NagZ: Increased Susceptibility of Gram-Negative Bacteria to β-Lactams," *ChemBioChem*, vol. 14, no. 15, pp. 1973–1981, 2013, doi: 10.1002/cbic.201300395.

[212] Q. Cheng and J. T. Park, "Substrate Specificity of the AmpG Permease Required for Recycling of Cell Wall Anhydro-Muropeptides," *J. Bacteriol.*, vol. 184, no. 23, pp. 6434–6436, Dec. 2002, doi: 10.1128/JB.184.23.6434-6436.2002.

[213] M. V, R. Km, G. S, and R. Di, "Crystallization and preliminary X-ray analysis of a UDP-MurNAc-tripeptide D-alanyl-D-alanine-adding enzyme (PaMurF) from Pseudomonas aeruginosa," *Acta Crystallograph. Sect. F Struct. Biol. Cryst. Commun.*, vol. 69, no. Pt 5, May 2013, doi: 10.1107/S1744309113005344.

[214] N. Rosenfeld, C. Bouchier, P. Courvalin, and B. Périchon, "Expression of the Resistance-Nodulation-Cell Division Pump AdeIJK in Acinetobacter baumannii Is Regulated by AdeN, a TetR-Type Regulator," *Antimicrob. Agents Chemother.*, vol. 56, no. 5, pp. 2504–2510, May 2012, doi: 10.1128/AAC.06422-11.

[215] R. Ago and D. Shiomi, “RodZ: a key-player in cell elongation and cell division in Escherichia coli,” *AIMS Microbiol.*, vol. 5, no. 4, pp. 358–367, Nov. 2019, doi: 10.3934/microbiol.2019.4.358.

[216] A. O. Henriques, P. Glaser, P. J. Piggot, and C. P. Moran Jr, “Control of cell shape and elongation by the rodA gene in Bacillus subtilis,” *Mol. Microbiol.*, vol. 28, no. 2, pp. 235–247, 1998, doi: 10.1046/j.1365-2958.1998.00766.x.

[217] P. D. A. Rohs *et al.*, “A central role for PBP2 in the activation of peptidoglycan polymerization by the bacterial cell elongation machinery,” *PLOS Genet.*, vol. 14, no. 10, p. e1007726, Oct. 2018, doi: 10.1371/journal.pgen.1007726.

[218] P. D. A. Rohs and T. G. Bernhardt, “Growth and Division of the Peptidoglycan Matrix,” *Annu. Rev. Microbiol.*, vol. 75, no. 1, pp. 315–336, 2021, doi: 10.1146/annurev-micro-020518-120056.

[219] R. M. Figge, A. V. Divakaruni, and J. W. Gober, “MreB, the cell shape-determining bacterial actin homologue, co-ordinates cell wall morphogenesis in Caulobacter crescentus,” *Mol. Microbiol.*, vol. 51, no. 5, pp. 1321–1332, 2004, doi: 10.1111/j.1365-2958.2003.03936.x.

[220] T. T. Truong, A. Vettiger, and T. G. Bernhardt, “Cell division is antagonized by the activity of peptidoglycan endopeptidases that promote cell elongation,” *Mol. Microbiol.*, vol. 114, no. 6, pp. 966–978, Dec. 2020, doi: 10.1111/mmi.14587.

[221] C. Micelli *et al.*, “A conserved zinc-binding site in Acinetobacter baumannii PBP2 required for elongasome-directed bacterial cell shape,” *Proc. Natl. Acad. Sci.*, vol. 120, no. 8, p. e2215237120, Feb. 2023, doi: 10.1073/pnas.2215237120.

[222] K. N. Kang and J. M. Boll, “PBP1A Directly Interacts with the Divisome Complex to Promote Septal Peptidoglycan Synthesis in Acinetobacter baumannii,” *J. Bacteriol.*, vol. 204, no. 12, p. e0023922, Dec. 2022, doi: 10.1128/jb.00239-22.

[223] I. Shlosman *et al.*, “Allosteric activation of cell wall synthesis during bacterial growth,” *Nat. Commun.*, vol. 14, no. 1, Art. no. 1, Jun. 2023, doi: 10.1038/s41467-023-39037-9.

[224] A. J. Meeske *et al.*, “SEDS proteins are a widespread family of bacterial cell wall polymerases,” *Nature*, vol. 537, no. 7622, pp. 634–638, Sep. 2016, doi: 10.1038/nature19331.

[225] E. Oh, J.-Y. Zhu, M.-Y. Bai, R. A. Arenhart, Y. Sun, and Z.-Y. Wang, “Cell elongation is regulated through a central circuit of interacting transcription factors in the Arabidopsis hypocotyl,” *eLife*, vol. 3, p. e03031, May 2014, doi: 10.7554/eLife.03031.

[226] J. Errington and L. J. Wu, “Cell Cycle Machinery in Bacillus subtilis,” in *Prokaryotic Cytoskeletons: Filamentous Protein Polymers Active in the Cytoplasm of Bacterial and Archaeal Cells*, J. Löwe and L. A. Amos, Eds., in Subcellular Biochemistry. , Cham: Springer International Publishing, 2017, pp. 67–101. doi: 10.1007/978-3-319-53047-5_3.

[227] G. C. Lai, H. Cho, and T. G. Bernhardt, “The mecillinam resistome reveals a role for peptidoglycan endopeptidases in stimulating cell wall synthesis in Escherichia coli,” *PLoS Genet.*, vol. 13, no. 7, p. e1006934, Jul. 2017, doi: 10.1371/journal.pgen.1006934.

[228] B. W. Simpson, M. C. Gilmore, A. B. McLean, F. Cava, and M. S. Trent, “Escherichia coli utilizes multiple peptidoglycan recycling permeases with distinct strategies of recycling,” *Proc. Natl. Acad. Sci.*, vol. 120, no. 44, p. e2308940120, Oct. 2023, doi: 10.1073/pnas.2308940120.

[229] Y. Doi, “Treatment Options for Carbapenem-resistant Gram-negative Bacterial Infections,” *Clin. Infect. Dis.*, vol. 69, no. Supplement_7, pp. S565–S575, Nov. 2019, doi: 10.1093/cid/ciz830.

[230] A. Matlock, J. A. Garcia, K. Moussavi, B. Long, and S. Y.-T. Liang, “Advances in novel antibiotics to treat multidrug-resistant gram-negative bacterial infections,” *Intern. Emerg. Med.*, vol. 16, no. 8, pp. 2231–2241, Nov. 2021, doi: 10.1007/s11739-021-02749-1.

[231] “Updating Breakpoints in Antimicrobial Susceptibility Testing,” ASM.org. Accessed: Nov. 13, 2023. [Online]. Available: https://asm.org:443/Articles/2022/February/Updating-Breakpoints-in-Antimicrobial-Susceptibili

[232] A. I. Weaver *et al.*, “Lytic transglycosylases mitigate periplasmic crowding by degrading soluble cell wall turnover products,” *eLife*, vol. 11, p. e73178, Jan. 2022, doi: 10.7554/eLife.73178.

[233] A. Sharma, R. Sharma, T. Bhattacharyya, T. Bhando, and R. Pathania, “Fosfomycin resistance in Acinetobacter baumannii is mediated by efflux through a major facilitator superfamily (MFS) transporter—AbaF,” *J. Antimicrob. Chemother.*, vol. 72, no. 1, pp. 68–74, Jan. 2017, doi: 10.1093/jac/dkw382.

[234] I. Kyriakidis, E. Vasileiou, Z. D. Pana, and A. Tragiannidis, “Acinetobacter baumannii Antibiotic Resistance Mechanisms,” *Pathogens*, vol. 10, no. 3, p. 373, Mar. 2021, doi: 10.3390/pathogens10030373.