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# Molecular Mechanisms That Regulate the Stress Response In A. baumannii

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# Molecular Mechanisms That Regulate the Stress Response In *A. baumannii*

by

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# DEDICATION

To my wife and kids, whose sacrifices, love, and constant support have enabled me to reach my full potential and follow my dreams.

Approved by Supervisory Committee:

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# CHAPTER 1

## INTRODUCTION

Due to the increasing incidence of multidrug resistant Gram-negative microorganisms connected to deadly nosocomial infections, there is an urgent need to describe new antibiotic targets and design innovative antimicrobial treatment protocols in order to combat antibiotic treatment failure and limit the spread of resistance. A serious public health concern has been exacerbated by the rising incidence of antibiotic treatment failure. Classical treatment protocols no longer eradicate bacterial infections that were traditionally cleared quickly with antibiotics[1]. The Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO) continue to list nosocomial-associated *Acinetobacter baumannii* as an urgent threat to public health and also as a crucial target for antibiotic development, respectively[2,3]. The clinically important beta-lactam disrupts essential peptidoglycan biosynthesis and maintenance to provide antibacterial activity[4,5]. Carbapenem beta-lactams such imipenem and meropenem, were resistant to many common beta-lactam resistance mechanisms and were only used as a last option to treat multidrug resistant Gram-negative infections[6,7]. Meropenem is actually a last-resort carbapenem antibiotic for the treatment of multidrug-resistant Gram-negative infections[8,9]. However, *A. baumannii* is treated with meropenem initially, due to its extreme multidrug-resistance[10,11].

The rapid spread of *A. baumannii* strains resistant to not only carbapenems, but also other clinically important antibiotics, including beta-lactams, fluoroquinolones, tetracyclines, and aminoglycosides, makes therapy difficult[12,13]. Most strikingly, carbapenem-resistant *A. baumannii* has unfortunately become widespread in hospital-acquired infections and one remaining treatment option was colistin. Colistin is a cationic antimicrobial peptide (CAMP) that targets the Gram-negative outer membrane (OM)[14]. Colistin binds the phosphate groups on the lipid A anchor domain of lipooligosaccharide (LOS), which enables it to concentrate at the cell surface. Insertion and pore formation perturb the OM, which rapidly induces cell lysis[15,16]. Lipid A is synthesized by a highly conserved pathway where nine enzymatic steps produce a canonical

disaccharide that is fatty acylated and bis-phosphorylated[17]. Due to phosphorylation of the lipid A and core moieties in *A. baumannii*, an overall negative charge increases electrostatic potential with cationic (positively charged) antimicrobial peptides[18]. Importantly, *A. baumannii* can also rapidly develop resistance to polymyxin antibiotics via a unique resistance mechanism, complete inactivation of lipid A biosynthesis, which is the precursor to lipopolysaccharide (LPS)/LOS, which was canonically thought to be essential for Gram-negative bacteria survival[19]. Previous studies and the studies herein, suggest it is not[19,20]. The characterization of colistin-resistant *A. baumannii* isolates revealed that each had mutations in one of the first three lipid A biosynthesis pathway genes, *lpxA*, *lpxC*, or *lpxD*, which completely eliminated the ability to produce LOS[19,20]. While *A. baumannii* strains survive without lipid A biosynthesis to develop resistance to colistin and other clinically important antimicrobials, we do not understand the adaptive cellular responses or regulatory factors that promote survival of LOS deficient *A. baumannii*. It is also unknown how the OM of *A. baumannii* keeps its integrity in the absence of LOS.

Previous work from our group reported, BaeSR a two-component system (TCS) and lipoprotetins are highly upregulated in LOS deficient *A. baumanni*[20]. LOS deficient *A. baumannii* could not be recovered despite the genetic deletion of *baeSR* being dispensable for wild *type A. baumannii* survival, indicating that BaeSR controls a crucial function that promotes LOS survival. However, we don't know what signals activate BaeSR or what other gene products it regulates.

Here, we determined genes regulated by BaeSR two-component system. We found that BaeSR regulated phenyl alanine metabolism, a recently determined stress response of *A. baumannii*[21]. We also reported that OM lipoproteins contribute to the stiffness of LOS-deficient OM. OM lipoproteins are likely to be covalently attached to the peptidoglycan, which strengthens the cell envelope in the absence of LOS.

We also explored an innovative approach that could represent a potential therapeutic to combat *A. baumannii*. Ruthenium-based photosensitizers which have demonstrated activity against tumor cells[22], are also impressively active against *A. baumannii* in the low mM range. After screening several compounds, we identified seven with potent antimicrobial activity against multi-drug resistant (MDR) *A. baumannii*. Because we could not detect resistance,

photoactivated compounds could represent a possible alternative way to resolving the drug resistance phenomena.

Taken together, these studies provide insight into how *A. baumannii* maintains cell envelope homeostasis in the absence of lipid A/LOS. These stress response pathways will aid in the future design of the antibacterial treatment protocols and help to establish a development pipeline. Potent photo-activated antimicrobials with efficacy against *A. baumannii* could help us to counter *A. baumannii* infections

# CHAPTER 2

# LITERATURE REVIEW

## The Gram-negative cell envelope

The Gram-negative cell envelope is a complicated structure composed of three distinct layers, each with its own function. It differs from Gram-positive bacteria's cell envelope in that it has a second lipid bilayer, the outer membrane (OM), in addition to the inner membrane. The inner (cytoplasmic) membrane is a phospholipid bilayer that surrounds the bacterial cell's cytoplasm. It is selectively permeable, allowing chemicals to enter and exit the cell[23,24]. It contains proteins that are involved in cellular functions such as nutrient absorption, energy production, and macromolecule synthesis[23,24]. Between the inner and OM is the periplasmic space. It is a gel-like area containing numerous proteins, enzymes, and transport mechanisms[23–25]. The periplasmic space is critical for preserving cell integrity, safeguarding the inner membrane, and allowing extracellular processes to occur[23–26]. The periplasm houses the peptidoglycan, a mesh-like structure that protects and structurally supports the bacterial cell. It is made up of repetitive units of sugar chains connected by peptide cross-bridges[23–26]. The peptidoglycan in Gram-negative bacteria is thinner relative to Gram-positive bacteria. The OM is an asymmetric lipid bilayer made up of phospholipids, proteins, and surface-exposed lipopolysaccharide (LPS)[23,24,27]. The outer membrane acts as a barrier against potentially toxic compounds such as antibiotics and detergents[23,24,27]. Channels called porins allow small molecules like ions and nutrients to pass through.

LPS is composed of three regions: lipid A, core oligosaccharide, and O antigen[28]. Lipid A anchors LPS to the OM. LPS is a potent activator of the innate immune system. Due to its inflammatory potential, LPS is also called endotoxin[29]. LPS structural variations and antigenicity are dictated by the lipid A moiety[17].

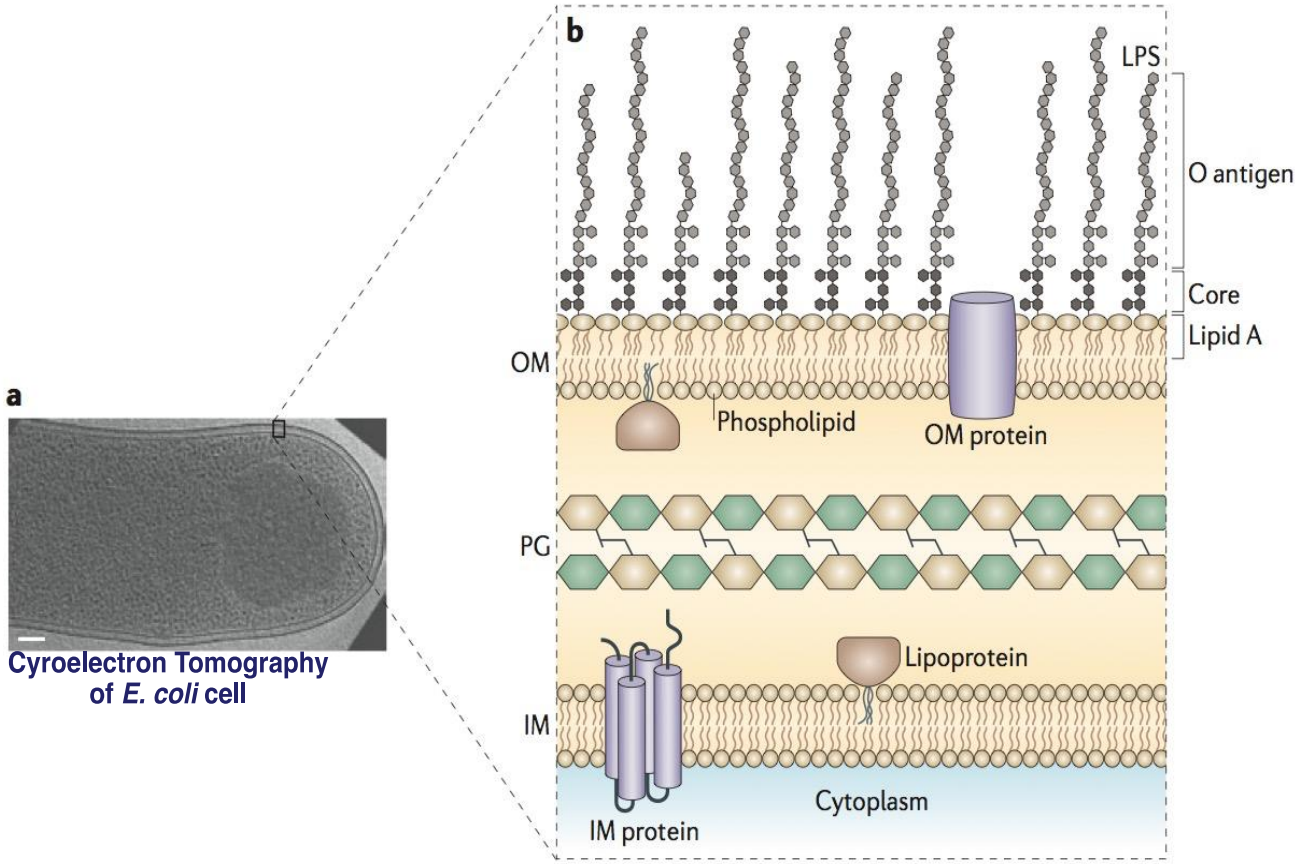



**Figure 1: Gram-negative cell envelope as described in Needham *et al* (2013)**[30]

## LOS-deficient (LOS$^-$) Gram-negative cell envelope

Gram-negative bacteria lacking the O-antigen domain of LPS, are called lipooligosaccharide (LOS)[19,31,32]. These strains were initially known as "rough" strains, as opposed to "smooth" strains that had a complete LPS structure[33]. The presence or lack of the O-antigen became an important criterion in the classification of Gram-negative bacteria. Researchers were able to detect and classify diverse strains by evaluating the reactivity of bacterial strains with antisera targeting different O-antigens. Over time, researchers concentrated their efforts on determining the genetic basis of LOS biosynthesis. Lipid A (endotoxin), the hydrophobic anchor of LPS/LOS, is a glycolipid that enriched in the surface monolayer of most Gram-negative bacteria[28]. The enzymology and conserved steps of lipid- A biosynthesis was described in *E. coli*[28]. Pathogens and many Gram-negative bacteria produce lipid A species similar to that found in *E. coli*[28]. Lipid A is synthesized by nine constitutive enzymatic pathway famously known as Raetz

pathway[17], named after the late Christian Raetz. The first three enzymes in the pathway are cytoplasmic LpxA, LpxC, and LpxD. The peripheral membrane proteins LpxH and LpxB catalyze the fourth and fifth steps of lipid A biosynthesis[34,35]. Integral membrane enzymes conduct the final four steps[36,37]. MsbA, an ABC transporter, flips $KDO_2$-lipid A from the inner leaflet of the inner membrane to the periplasmic leaflet of the inner membrane after synthesis[38,39]. Various glycosyltransferases attached core oligosaccharide followed by O-antigen ligation to produce LPS, which is subsequently transported to the surface of the cell[40]. *A. baumannii* does not encode and O-antigen ligase and can also rapidly acquire resistance to polymyxin antibiotics due to total loss of the original binding target, lipid A. This research added to our knowledge of the genetics and regulation of LOS biosynthesis. Researchers also investigated the effect of LOS in Gram-negative bacteria pathogenicity. LOS was discovered to be critical in bacterial survival, colonization, and immune evasion[30,41,42]. LOS-deficient strains were frequently associated with decreased virulence and altered host interactions, emphasizing the importance of LOS in bacterial pathogenesis .

Mutations or changes in the genes responsible for lipid A biosynthesis can cause LOS absence in Gram-negative bacteria[19,31,32]. This can have serious consequences for bacterial pathogenicity and the host immunological response. The structural stability of the OM is dependent on LOS. The integrity of the OM can be impaired in its absence or inadequacy, resulting in decreased fitness and survival of the bacteria[43,44]. This can limit growth and colonization abilities. The presence of LOS can operate as a barrier to antibiotic penetration, imparting resistance to the bacteria. LOS is essential in the pathogenesis of Gram-negative bacteria. It has the ability to engage the host immune system and induce the production of pro-inflammatory cytokines[20,45,46]. LOS deficiency can impair bacteria's capacity to engage the immune system, resulting in a diminished virulence phenotype[43,44].

LOS-deficient strains may be less recognized by host immune cells such macrophages and neutrophils, which predominantly recognize and respond to lipid A[31,47–51]. This can lead to a less efficient clearance of bacteria by the immune system and a longer infection. LOS is also used as a marker in Gram-negative bacteria identification tests[52]. In these tests, LOS-deficient bacteria may exhibit unusual or mild reactions, posing diagnostic problems. It should be noted that LOS

deficiency can occur spontaneously in certain bacterial strains or be produced in the laboratory . LOS-deficient mutants are frequently used by researchers to investigate the role of LOS in bacterial pathogenesis and the host immunological response. Overall, research into LOS deficiency has helped us understand the structure, function, and toxicity of Gram-negative bacteria. It has generated insights into bacterial genetics, serotyping, immune evasion techniques, and diagnostic method development. Research on LOS-deficient strains and their interactions with the host immune system is still ongoing in microbiology and infectious diseases.

**_A. baumannii_, a nosocomial pathogen**

*A. baumannii* is a Gram-negative coccobacillus, non-motile, catalase-positive, oxidase-negative, non-fastidious and strictly aerobic bacterium and one of the most prevalent causes of nosocomial infections [54]. The expanding resistance of *A. baumannii* to primary antimicrobial therapies has created a deadly combination of pathogenicity and antimicrobial resistance that plagues hospitals [55]. Categorized as an ESKAPE pathogen (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, A. baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species), carbapenem-resistant *A. baumannii* is considered the World Health Organization's number one critical priority pathogen for which new therapeutics are urgently needed [56]. Therefore, without a significant intervention, hospital-acquired *A. baumannii* infections will soon be untreatable.

Dutch bacteriologist Beijernick introduced *A. baumannii* in 1911 and originally named it *Micrococcus calcoaceticus*[57]. Interestingly, the same bacterium was isolated many times and reported with various names such as *Moraxella lwoffi*, *Alcaligenes hemolysans*, *Mirococcuscalco-aceticus*, and *Herellea vaginicola.* Forty years later Brisou and Prevot included this organism in the *Acinetobacter* genus based on its non-motile and colorless property[58]. In 1972 the committee on the taxonomy of Moraxella and Allied Bacteria accepted the inclusion of all such isolates in one genus, *Acinetobacter*[59]. Bouvet and Grimont further classified the organism into twelve groups based on DNA similarity, which were taxonomically classified as γ-proteobacteria, family Moraxellaceae and order Pseudomonadales in late 80s[60]. Later on, it was reclassified into more than fifty different species and the scientific community faced significant challenges in differentiating species within *A. baumannii-calcoaceticus* (ABC) complex, which includes *A.*

*baumannii*, *A. calcoaceticus*, *A. nosocomialis*, and *A. pittii*, with *A. baumannii* deemed as the most virulent one[61]. *A. baumannii* has been isolated from various body parts of healthy individuals, including the nose, ear, throat, forehead, trachea, conjunctiva, vagina and perineum, axillae, groin, hands, and toe webs[62]. More alarmingly, *A. baumannii* has been isolated primarily from hospitals, where the organism inhabits beds, curtains, walls, roofs, medical devices, and equipment, as well as on belongings of medical personnel, tap water sinks, telephones, door handles, hand sanitizers, dispensers, trolleys, bins, and even on electronic devices. The underlying cause of their persistence in the hospital environment is linked with their resistance to key antimicrobial drugs and disinfectants and their ability to endure desiccants[63]. Despite, the current resurgence in antimicrobial research and development, *A. baumannii* remains the most important unmet medical need among resistant Gram-negative pathogens.

**Clinical Significance of *A. baumannii***

*A. baumannii* poses a significant public health concern as its emerged as a major culprit in hospital-based infections, especially in intensive care units (ICUs) worldwide. The potential of these organisms to pollute the hospital is linked with nosocomial outbreaks[64]. *A. baumannii* causes a wide variety of infections in hospitals as well as in the community which includes skin and soft tissue, urinary tract infections, meningitidis, bacteremia, and pneumonia[65]. Critically ill patients are most commonly infected by hospital-based infections; major predisposing factors for developing an *A. baumannii* infection include prolonged hospital stays, immune suppression, advanced age, presence of comorbid diseases, major trauma or burns, previous antibiotic use, invasive procedures, and presence of indwelling catheters or mechanical ventilation[66–69]. Because of the already poor prognosis of critically ill patients who acquire *A. baumannii* infections, it is difficult to attribute a definitive mortality rate the rate goes up in ICUs [70]; however, roughly 23 to 68% infected people die[10].

**Treatment options against *A. baumannii***

Due to extensive ability to develop resistance through intrinsic and acquired mechanisms, we are running out of options to treat *A. baumannii* infections. This bacterium is endowed with genetic setup for rapid development of antimicrobial resistance. Until 1975, *Acinetobacter* infections were treated with ampicillin, carbenicillin, gentamicin and nalidixic acid, either as

monotherapy or combination therapy. However, after 1975 prescriptions were altered due to high rates of resistance . Currently, carbapenems (imipenem and meropenem) are viable options to treat *Acinetobacter* infections, but recently resistance rates increased substantially[63]. Colistin (polymyxin E) is the last line treatment option for patients harboring carbapenem resistance isolates[63]. Nevertheless, colistin resistant isolates have emerged[72]. Aside from carbapenems and colistin, aminoglycosides (amikacin and tobramycin), fluroquinolones (ciprofloxacin and levofloxacin), broad-spectrum cephalosporins (ceftazidime, cefotaxime, ceftriaxone, cefepime), glycylcyclines (tigecycline) and combinations of β-lactamase inhibitors with antibiotic (ampicillin/sulbactam) are currently prescribed, provided the organisms are susceptible; though minimum inhibitory concentrations (MICs) have noticeably increased[61]. Due to the decline in susceptibility to variety of antibiotic agents, combinational therapies are introduced where multiple antibiotic agents are applied for synergistic effects.

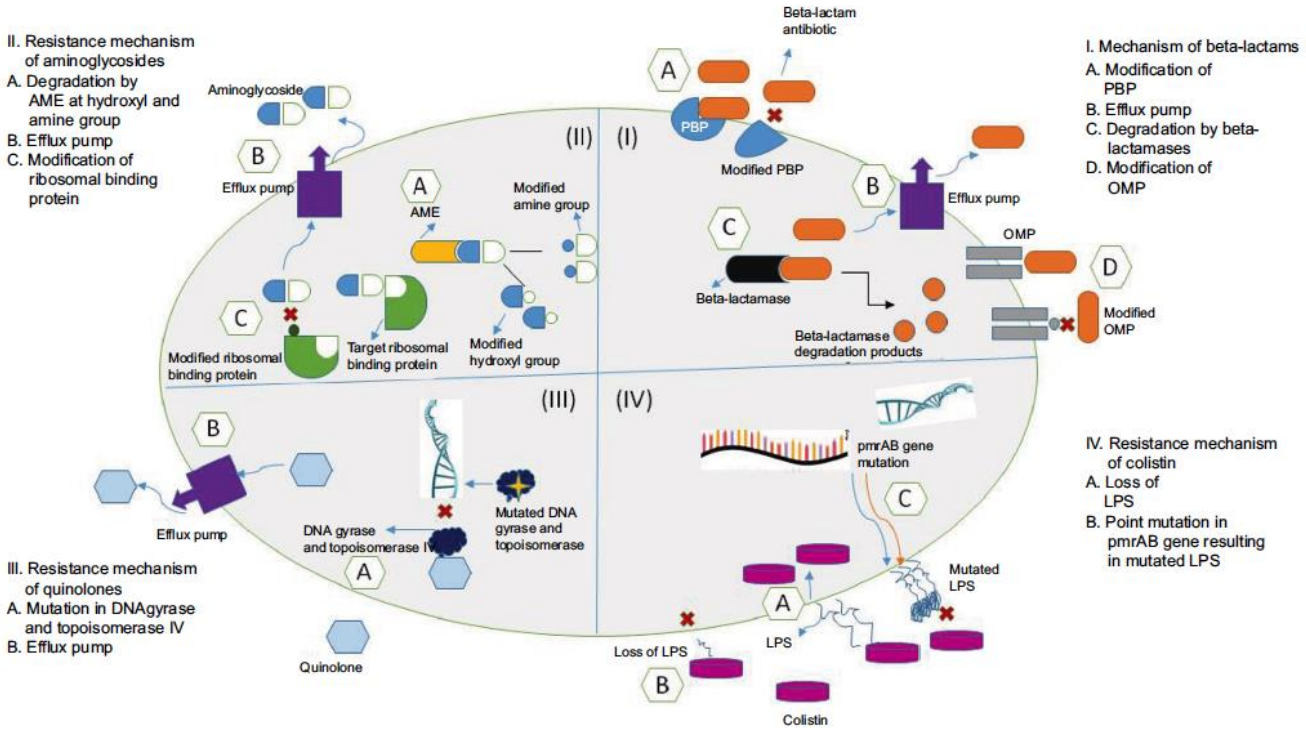



**Figure 2: Mechanisms of resistance in *A. baumannii*, (I) β-lactams; (II) aminoglycosides; (III) quinolones; (IV) colistin as described in Asif *et al* (2018)[73].**

Abbreviations: AME, aminoglycoside modifying enzyme; LPS, lipopolysaccharide; OMP, OM porin; PBP, penicillin-binding protein

## ***A. baumannii*** **develops resistance against last resort antibiotics**

OM perturbations trigger cellular leakage and death, a property that makes the conserved structure a target for therapeutic treatment[74]. For example, colistin is a cationic antimicrobial peptide (CAMP) that targets the Gram-negative OM[14]. Colistin binds the phosphate groups on the lipid A anchor portion of LOS, which enables it to concentrate at the cell surface. Insertion and pore formation perturb the OM, which rapidly kills the bacterium . *A. baumannii* thrives in hospitals because it is highly resistant to desiccation[20], biocide treatments[75], and rapidly develops resistance to a wide range of antimicrobials[10]. Occurrences of MDR *A. baumannii* infections have soared over the past decade and one remaining treatment option was colistin.

**Surprisingly, *A. baumannii* encodes mechanisms to overcome colistin treatment through mutation of the lipid A biosynthetic pathway, which inactivates LOS assembly**[19,20]**.** LOS$^-$ *A. baumannii* demonstrated an increased minimal inhibitory concentration (MIC) to colistin, measuring 0.5 in wild type and >256 μg/ml in the LOS$^-$ mutant[20] **(Figure 3)**. Since clinical resistance is defined as approximately 4 μg/ml, this resistance level could easily promote *A. baumannii* growth in colistin-treated hosts . Moreover, LOS$^-$ *A. baumannii* was isolated from patients after colistin treatment[77]. LOS$^-$ *A. baumannii* also demonstrated MDR to other commonly prescribed treatment options[20] **(Figure 3)**.

While *A. baumannii* strains survive without lipid A biosynthesis to develop resistance to colistin and other clinically important antimicrobials, we do not understand the adaptive responses or regulatory factors that promote survival of LOS$^-$ *A. baumannii*. Our previous work[20] and a more recent transcriptomics study suggested that mutational inactivation of the lipid A biosynthetic pathway did not significantly alter the cellular glycerophospholipids (GPL) ratios. While GPLs were previously hypothesized to mechanistically replace LOS in the outer leaflet of the OM[31,79], overexpression of the ***m***aintenance of the OM ***l***ipid ***a***symmetry (*mla*) pathway, which prevents cell surface GPL accumulation, likely maintained asymmetry[20,78,80]. This cellular response suggests that LOS$^-$ cells utilize a separate compensatory mechanism to replace LOS in the surface-exposed leaflet of the OM.

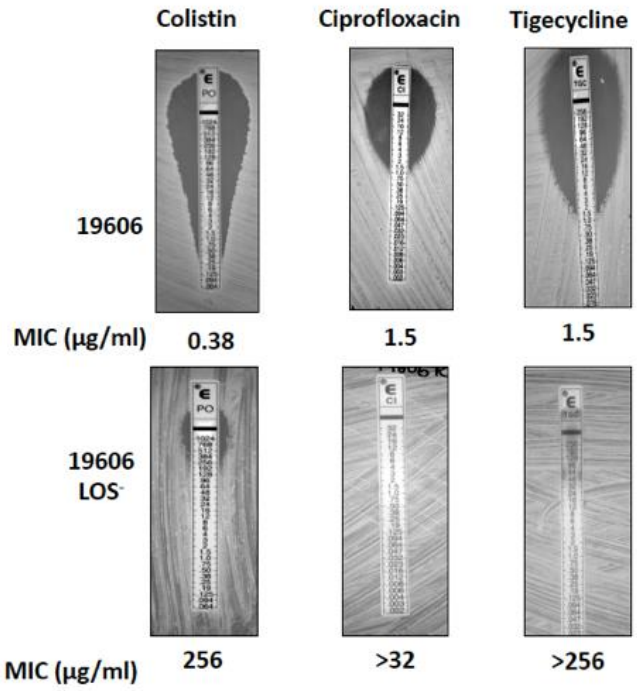



**Figure 3: Minimum Inhibitory concentrations (MIC) in wild type and LOS deficient ATCC 19606 *A. baumannii* as described in Boll et all (2016)**[20]

## How *A. baumannii* respond to LOS deficiency in their physiology

*A. baumannii* uses a range of coping mechanisms in response to stressful situations. The bacterium can adapt, endure, and persist in many conditions because to these stress response systems. To understand cellular alterations in LOS$^-$ *A. baumannii*, we examined the transcriptome of wild type and LOS$^-$ *A. baumannii*. A conserved response was found in multiple strains and isolates, where five pathways were overexpressed in the LOS$^-$ mutants relative to the wild type parent strain **(Figure 4)**. Pathways that belong to this conserved response are: 1) Lipoprotein transport pathway 2) Putative Lipoproteins 3) Multidrug efflux pumps 4) BaeSR two component system 5) phospholipid transport. Interestingly all of these pathways are part of envelope maintenance[80–85].

*A. baumannii* has several two-component systems that are involved in detecting and reacting to stress signals, including AdeRS, BaeSR, BfmRS and PmrAB. These TCS control the expression of genes that help the organism adapt to stress. For instance, although BaeSR is implicated in the response to envelope stress and antimicrobial peptides, the AdeRS system

regulates the development of the AdeABC efflux pump, which contributes to antibiotic resistance [81,86]. There are still few studies on the environmental cues that BaeSR responds to. In *E. coli,* BaeSR regulates a small regulon that contains essential efflux pumps. The subsequent increase of efflux pumps supports a similar function[83]. However, efforts to identify the DNA binding sites in the promoters of the reported target genes have been unsuccessful, leaving room for further investigation[83]. BaeSR TCS in *Acinetobacter* is homologous to TCSs in *E. coli* and *Salmonella enterica* serovar Typhimurium based on sequence similarity[18]. *baeSR* transcription is induced as an adaptive response when *A. baumannii* shuts down LOS[20] biosynthesis. Furthermore, while genetic deletion of *baeSR* was dispensable for wild type *A. baumannii* survival, LOS$^-$ *A. baumannii* could not be recovered, suggesting BaeSR regulates some essential function that supports LOS$^-$ survival.

The deficiency of LOS results in different physical and chemical characteristics of the Gram-negative cell envelope that affects membrane biogenesis and physiology[78] and various -omics approaches were introduced to unveil these changes all together. Upon sensing stress or membrane perturbations, Gram-negative bacteria respond by modifying the cell envelope. In *E. coli* at least five envelope stress response pathways including Psp (Phage shock protein), Bae (bacterial adaptive response), Cpx (conjugative plasmid expression), Rcs (regulator of capsule synthesis), and Ó$^E$ induce modifications under various adverse conditions[87]. However, *A. baumannii* encodes only three homologous systems, including Psp, Bae, and Cpx systems[88]. *A. baumannii* also encodes the AdeSR TCS, which is thought to overlap with the BaeSR regulon[89]. While the regulatory targets of these TCSs have not been extensively characterized in *A. baumannii*, they likely regulate cell envelope stress responses. Furthermore, the BaeSR TCS was essential for LOS$^-$ *A. baumannii* survival, suggesting some regulatory product is essential.

The *A. baumannii* OM serves as a defense against numerous stressors[24]. The expression and make-up of outer membrane proteins (OMPs) can be changed by the bacteria in response to stress in order to adapt and defend itself. *A. baumannii* may be able to withstand antimicrobial agents and get around the host immune system thanks to this regulation of OMPs [90]. Lipoproteins are included in the outer membrane's inner leaflet and make covalent linkages with the periplasmic peptidoglycan layer in addition to LPS/LOS and phospholipids[91]. The Braun's

lipoprotein (Lpp), which is the most prevalent lipoprotein in *E. coli*, is assumed to contribute to the stability and integrity of the outer membrane by covalently attaching it to the peptidoglycan cell wall. These preliminary findings indicate a mechanism to maintain an asymmetrical OM, where lipoproteins are enriched in the outer leaflet of the LOS$^-$ OM and the inner leaflet is composed of GPLs. LOS-deficient *A. baumannii* upregulate both the *lol* and *mla* pathways which is responsible for anterograde lipoprotein and retrograde phospholipid transport respectively[80,92]. Our preliminary work has found that lipoproteins are anchored to the cell surface-exposed leaflet in the LOS$^-$ OM[20]**.** Consistent with this hypothesis, a recent study showed that lipoprotein expression was not diminished over 120 generations in LOS$^-$ strains evolved to improve fitness, suggesting that surface-exposed proteins play a specific and important role in the LOS$^-$ cell envelop.

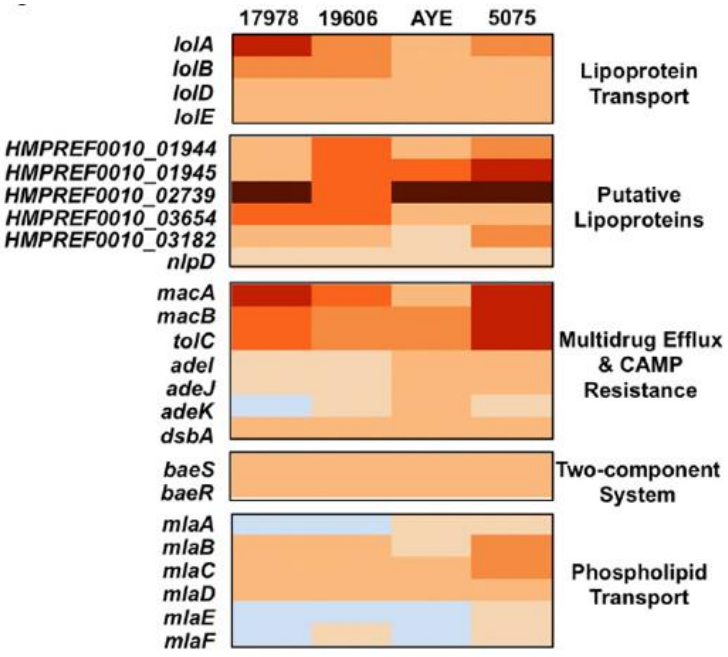



**Figure 4: Heatmap illustrating the altered expression of each gene in the five conserved pathways from multiple LOS$^-$ *A. baumannii* strains compare to their parent as described in Boll et all (2016)** [20]

The ability of *A. baumannii* to endure and persist under a variety of stress circumstances, such as those present during an infection and antibiotic treatment, is largely attributed to these response mechanisms. They also support the bacterium's capacity to produce chronic infections

and antibiotic resistance. It's imperative to comprehend these mechanisms of stress response in order to create efficient defenses against *A. baumannii* infections.

**Bacterial two component system (TCS)**

A regulatory mechanism frequently seen in bacteria that enables them to detect and react to changes in their environment is known as a bacterial two-component system (TCS)[93,94]. A sensor kinase and a response regulator are its two major parts **(Figure 5a)**. The sensor kinase often spans the membrane of the bacterial cell and is membrane-bound. It has an extracellular sensing domain that can pick up on particular environmental cues including temperature, pH, the availability of nutrients, osmolarity, or the presence of particular chemicals[95]. The sensor kinase's intracellular domain functions as a kinase that can phosphorylate itself in response to the signal that is being detected. The response regulator, on the other hand, normally receives the phosphoryl group from the sensor kinase and is a cytoplasmic protein. The response regulator goes through a conformational shift after being phosphorylated, which makes it possible for it to bind to DNA sequences known as promoters or operator regions. The response regulator can either activate or inhibit the transcription of target genes by binding to these regulatory areas.

In conclusion, the sensor kinase autophosphorylates and transfers the phosphoryl group to the response regulator in response to a particular environmental signal. When a signal is identified, the phosphorylated response regulator modifies gene expression to cause the proper cellular response. By regulating the expression of genes involved in functions like metabolism, pathogenicity, motility, and stress responses, this regulatory mechanism enables bacteria to adapt and survive in a variety of situations[89,96]. Bacteria have a vast variety of two-component systems, which are essential to their pathogenicity, adaptation, and survival. As disrupting their signaling pathways can impair bacterial growth and pathogenicity, they are crucial targets for research into the physiology of bacteria and the development of antimicrobial methods[97]. *A. baumannii* has several well characterized two-component systems (TCS) that help with its adaptability, pathogenicity, and resistance to antibiotics. **Figure 5b** illustrated the known TCS in *A. baumannii* and their physiological functions.

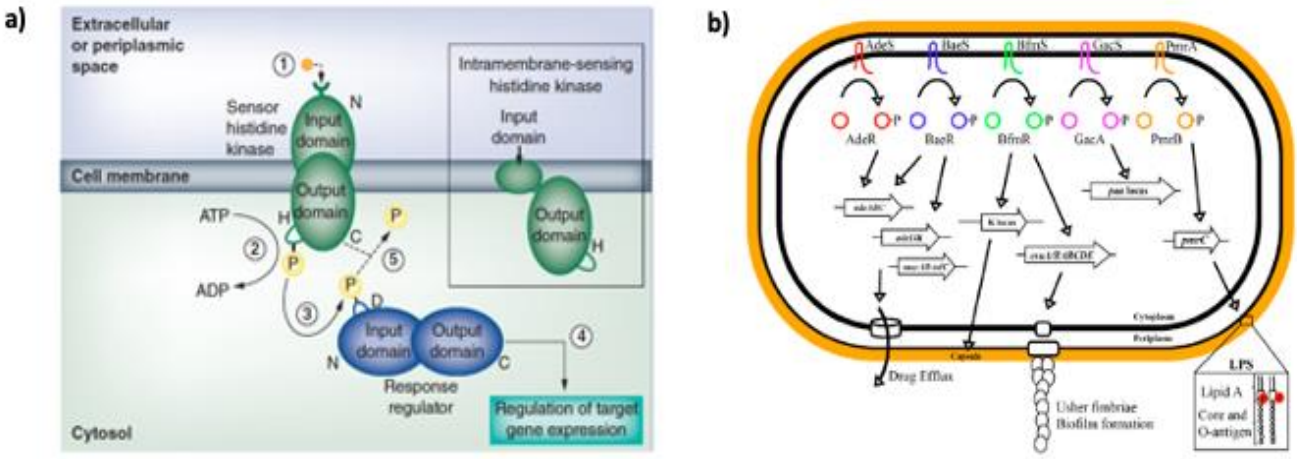



**Figure 5: Two component system, a) The basic process of two-component signal transduction[98] and b) Overview and impact of known TCS in *A. baumannii* [89] as described in Teierney et al (2019) and Kröger et al (2016)**

## Phenylacetic acid (PAA) catabolism mediates a stress response pathway of *A. baumannii*

Microorganisms can catabolize the substance phenylacetic acid (PAA) through a variety of routes. Conversion of PAA to phenylacetyl-CoA, which can subsequently enter the central metabolic pathways for further degradation, is one typical mechanism for PAA catabolism. Depending on the organism and its metabolic capacity, the specific enzymes and intermediates involved in the catabolism of phenylacetic acid may change. It is known that some bacteria, including specific strains of *Escherichia coli* and Pseudomonas species, have genes and enzymes necessary for the breakdown of phenylacetic acid [99–102]. The ubiquitous aromatic chemical phenyl acetic acid (PAA) can be found in a variety of natural settings, including soil, water, and plant tissues. As a metabolic byproduct, it can also be generated by some microorganisms. The monoamine oxidase encoded by *mao* and the phenylacetaldehyde dehydrogenase encoded by *feaB* are two enzymes involved in the conversion of phenylalanine to PAA[103]. The breakdown of PAA into smaller molecules, which can then be further digested and used to produce energy, is referred to as catabolism. The β-ketoadipate pathway is a ubiquitous process, albeit the catabolic pathway of PAA differs between various animals. Through this mechanism, PAA is transformed into phenyl acetyl-CoA, which is then processed to create acetyl-CoA and succinyl-CoA via the β-ketoadipate intermediate. Overall, the catabolism of PAA is an important process for the

degradation of aromatic compounds in the environment and plays a significant role in the carbon cycle. The general response may be summed up as follows:

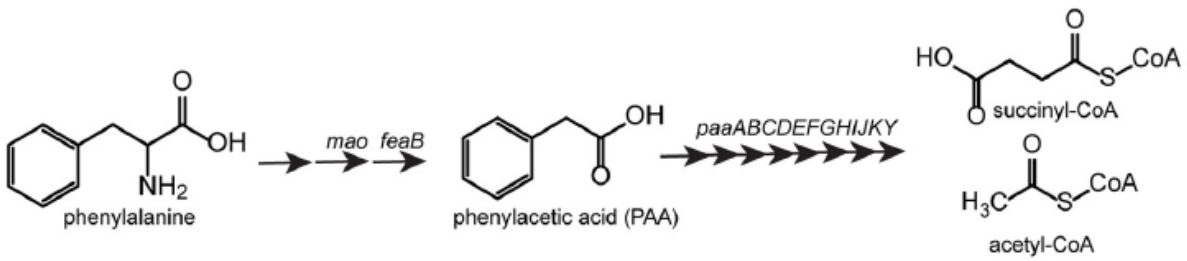



**Figure 6: Schematic of phenylalanine and PAA catabolism as described in Hooppaw *et al* (2022)**[21]

According to several transcriptome analyses carried out with different *A. baumannii* strains, the *paa* operon is one of the most differentially regulated pathways in response to various environmental conditions[104–108]. Even though metabolic pathways are routinely regulated in response to environmental changes, the PAA route is the only amino acid catabolic pathway in *Acinetobacter* that is so consistently and highly controlled throughout a wide range of environmental conditions. Studies have connected the *paa* operon to the pathogenicity of a variety of Gram-negative bacteria, including *Burkholderia cenocepacia* and *A. baumannii*. Inhibition of the upper PAA catabolic pathway (*paaABCDE*), for example, reduces the virulence of *B. cenocepacia* in a *Caenorhabditis elegans* model[109–112]. The *paa* operon, connected to pathogenesis in murine septicemia and zebrafish infection models, is regulated by the virulence-associated two-component system (TCS) GacSA in *A. baumannii* [110,111]. The lower pathogenicity of the Δ*paaA* strain in zebrafish was attributed to increased levels of secreted PAA, which serves as a neutrophil chemoattractant[110]. Although nothing is known about PAA's involvement in *Acinetobacter*, this knowledge suggests that it may be an important signaling molecule in several bacteria.

**Lipoproteins compensate for LOS deficiency in *A. baumannii* OM**

In addition to overexpression of the BaeSR two-component system genes, overexpressed pathways included, localization of lipoprotein (Lol) transport genes, maintenance of OM lipid asymmetry (Mla) genes, and five genes encoding lipoproteins (HMPREF0010_01944, 01945, 02739, 03654, and NlpD). Additional studies described herein as preliminary data indicate that

these pathways function in a coordinated response to decorate the surface-exposed leaflet of the LOS deficient OM with lipoproteins to maintain asymmetry and enable *A. baumannii* survival. The Lol transport system is an essential transport system composed of five proteins that shuttle fatty acylated proteins from the inner to OM in Gram-negative bacteria[92]. LolCDE assembles an ATPase that provides the energy for lipoprotein extraction from the inner membrane[113]. LolA is a periplasmic chaperone that binds and delivers lipoproteins to the OM acceptor protein LolB, which inserts them into the OM[114]. Based on this conserved model and our transcriptomic analysis, we *hypothesize* that the five lipoproteins are transported to the LOS$^-$ OM by the Lol system where they are flipped to the surface-exposed face via an unknown mechanism to replace LOS in the OM. To test this hypothesis, we examined OM proteins in LOS$^-$ *A. baumannii* (**Figure 7)**

Historically, Gram-negative inner and OM fractionations were performed using sucrose gradient ultracentrifugation, where bilayer separation is dependent on varied densities between the inner and OM lipid components [74,115]. However, we have been unable to effectively separate the bilayers in LOS- *A. baumannii* OM, presumably because the densities are roughly equivalent when LOS is removed from the OM. Despite this difficulty, we labeled surface-exposed proteins in wild type and LOS- whole cells using NHS-LC-LC-biotin, which does not cross the OM lipid bilayer [20,116,117]. An immunoblot of biotinylated surface-exposed proteins demonstrates distinct surface-exposed protein profiles between the wild type vs. LOS- strains (**Figure 7**). Additional protein bands in the membrane fraction of LOS- *A. baumannii* were confirmed to be three of the five overexpressed lipoproteins (01944, 01945 and 02739) (**Figure 7**).

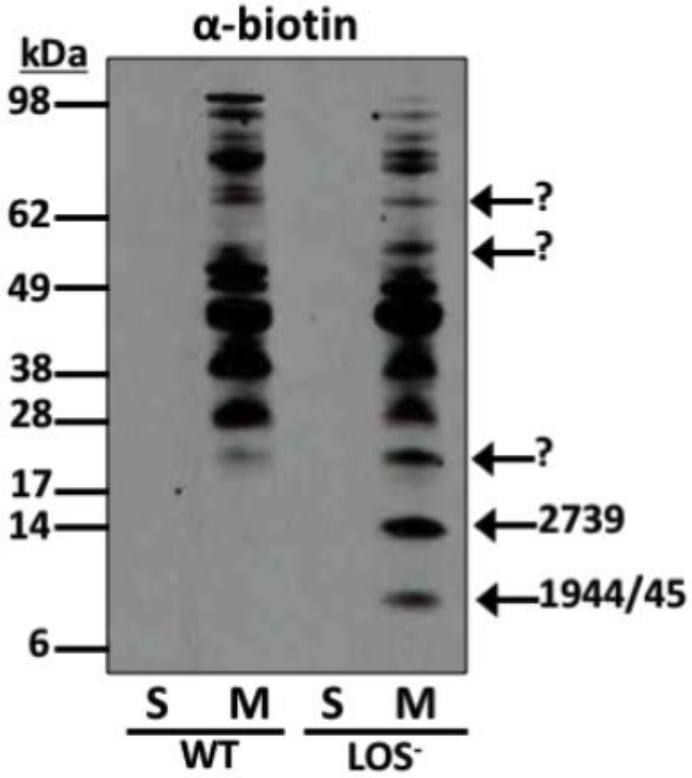



**Figure 7: Identification of surface displayed OM proteins. Whole *A. baumannii* cells were treated with sulfo-NHS-LC-LC biotin to biotinylate surface-exposed proteins, Soluble (S) and total membrane (M) compartments were fractionated after labeling, Previously identified proteins are labeled as described in Boll *et al* (2016)[20].**

## Photoantimicrobials an alternative to conventional antibiotics?

A group of substances known as photo-activated antimicrobials are those that have antibacterial qualities and can be stimulated or activated by light. Through a photochemical process, they are intended to fight microbial illnesses or stop microorganism growth. Utilizing photosensitizers, which are molecules with the ability to absorb light energy and transfer it to nearby molecules to produce reactive oxygen species (ROS) or other harmful species, is a common practice in photoantimicrobials[118] **(Figure 8)**. These ROS can harm the biomolecules and cellular architecture of microbes, which can kill or inhibit them[119,120]. The photosensitizer is exposed to light at a certain wavelength that matches the absorption spectra of the substance. The photosensitizer is excited by this energy to a higher energy state. By transferring energy to molecular oxygen, the excited photosensitizer can create singlet oxygen or other reactive oxygen species. These highly reactive species have the potential to harm microbial cells. The resulting

reactive species interact with lipids, proteins, and nucleic acids in cells, causing oxidative damage and impairing vital physiological functions[119,120].

In antimicrobial applications, photo-antimicrobials have various benefits. Since light may be directed at particular locations or provided using specialist equipment, they can offer localized and targeted treatment. They can also be triggered by non-invasive light sources, including LEDs or lasers, giving the treatment more precise control. Since photo antimicrobials work differently from conventional antibiotics in terms of how they work, using them may also help prevent the emergence of antibiotic resistance[121]. Scientists are constantly investigating new photosensitizers and light sources to improve the effectiveness and scope of photoantimicrobial research. They have demonstrated promise in processes like wound healing, surface sanitation, and the management of localized infections. Although photoantimicrobials have promise, more research is necessary to maximize their potency, ensure their safety, and assess their long-term effects.

Ruthenium complexes have drawn a lot of interest as photosensitizers because of their distinctive photochemical characteristics and prospective uses in a variety of industries. Ruthenium compounds are suitable as photosensitizers because they have advantageous photophysical characteristics[122]. They often absorb visible or near-infrared light, which allows for effective light energy consumption. Since ruthenium complexes frequently experience prolonged excited states, they can engage in a variety of photochemical activities, such as energy transfer and electron transport. Ruthenium complexes can act as photosensitizers in a variety of ways. One typical process is the energy transfer from the photoexcited ruthenium complex to an acceptor molecule. This can result in several photochemical events, such as the production of reactive oxygen species in photodynamic treatment. Another method, which can be applied in catalytic or redox processes, includes the transfer of electrons from the excited ruthenium complex to another molecule**.** Ruthenium complexes have showed potential in photodynamic therapy (PDT), a medical treatment that uses light-activated photosensitizers to kill cancer cells or microbial infections[123,124]. Ruthenium complexes' long-lived excited states allow efficient energy transfer to molecular oxygen, resulting in the formation of reactive oxygen species that can cause cell death or damage. Ruthenium complexes can operate as photochemical catalysts

in a variety of processes. They can, for example, be used in photochemical water splitting to generate hydrogen as a clean and renewable energy source. Ruthenium complexes can also be used in organic transformations such as photocatalytic oxidation processes or the creation of carbon-carbon bonds.

Light-responsive materials like photovoltaics, light-emitting diodes (LEDs), and sensors have included ruthenium complexes. They are useful components in these applications due to their effective light absorption and controllable photochemical characteristics. To investigate the mechanisms of photoinduced processes, photophysics, and photochemistry, ruthenium complexes are extensively researched in basic research. Their distinct characteristics and clearly defined structures make it possible for researchers to study light-matter interactions and comprehend the basic principles of photochemistry. Ruthenium complexes provide a flexible framework for creating photosensitizers with specialized characteristics for uses. In numerous sectors, such as medicine, energy, and materials science, research is now being done to better understand their potential and maximize their performance.

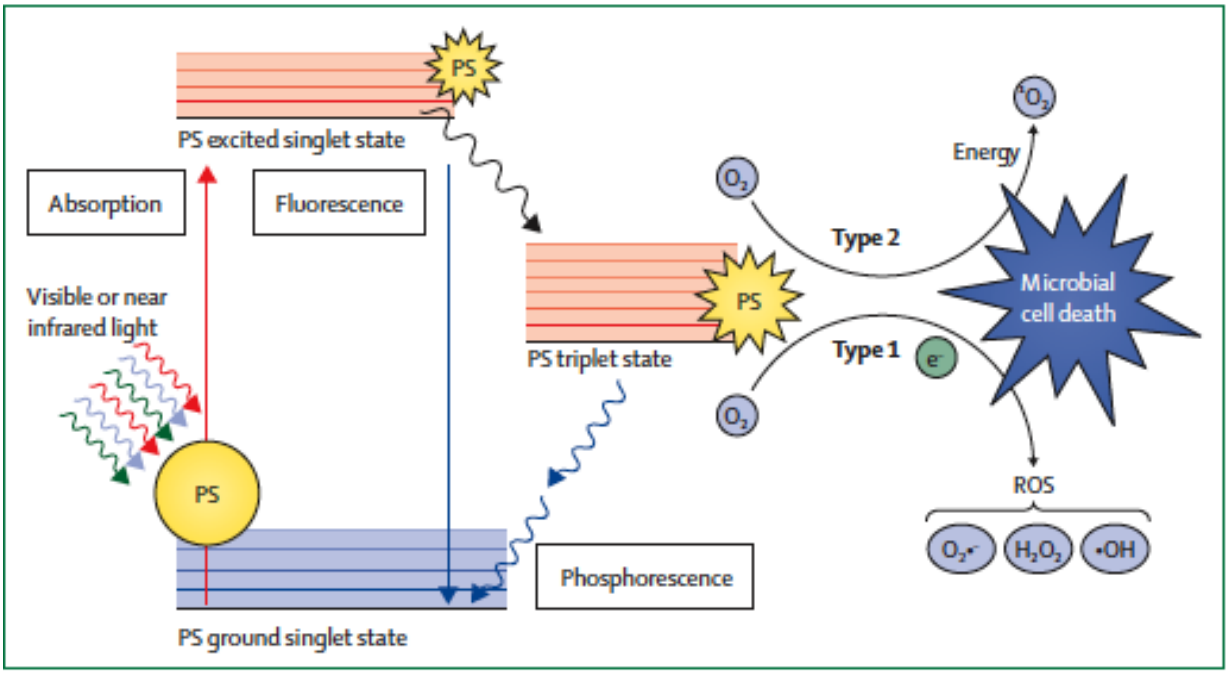



**Figure 8: Mechanism of photoantimicrobial action as described in Wainright *et al* (2017)** [118]

## CHAPTER 3

## BaeR directly regulates an *A. baumannii* stress response pathway


### Abstract

*Acinetobacter baumannii* is particularly problematic in hospital settings, where infections can occur in a range of tissues. This bacterium can endure extreme stress from various antimicrobial agents, biocides, host immune response etc. Not much is known about how these bacteria react to such high stress. *A. baumannii* also possesses a unique ability to inactivate lipooligosaccharide (LOS) biosynthesis, which is an essential molecule in most Gram-negative bacteria. LOS is enriched in the outer membrane and is the targeted by the last-resort antibiotic, colistin. LOS-deficient *A. baumannii* is highly resistant to the list-line antimicrobial. Previously, our lab discovered that a two-component system, called BaeSR, showed increased transcription in LOS-deficient cells relative to wild type. Furthermore, *baeSR* was required for LOS-deficient *A. baumannii* viability, suggesting that it regulated expression of some essential product. To determine BaeSR-dependent regulatory products we performed transcriptomics analysis on wild type and *baeR* mutants. Several pathways were downregulated in the mutant, including putative lipoproteins, lipoprotein transport genes, efflux pump genes, genes that regulate biofilm formation and phenylacetic acid catabolism. The *paa* operon acts on phenylacetic acid (PAA), an intermediary in the breakdown of phenylalanine, and is differently regulated in stress-induced conditions. Previous work showed that the GacSA two-component system also regulates the *paa* operon, but it was unknown if this regulation was direct or indirect. Notably, the sensor kinase, GacS*,* and response regulator, GacA*,* are not co-transcribed, which raises the possibility that the sensor kinase, GacS*,* may signal through other response regulators. We found that BaeR complementation not only restored wild type *paa* expression levels in Δ*baeR* and Δ*gacS*, but IPTG-dependent overexpression further induced *paa* overexpression. Electrophoretic mobility shift assay showed that the *paa* promoter bound recombinant $BaeR_{6X\text{-}his}$, suggesting a direct interaction. We also found that BaeR regulates expression of *csu* operon genes that controls biofilm formation. Biofilm formation is considered a stress response which relies on production of extracellular polymeric substances (EPS) to provide structural support so bacterial pathogens

can withstand adverse conditions. This data presented here in suggest the BaeSR TCS contributes to *A. baumannii* survival in stress.

**Introduction**

Antimicrobials, host immune response, osmotic pressure, and nutritional limitation are all examples of unfriendly environmental challenges that Gram-negative bacteria encounter in nature[125]. Bacteria sense and respond to stress with protective mechanisms to counter adverse environmental conditions[126]. Bacterial two-component systems stress specific signals and induce responses that can repair cellular damage by altering cellular metabolism, protein function, and gene expression[125,127] . *Acinetobacter baumannii* is an opportunistic Gram-negative bacterium, has a special ability to resist harsh environments and is responsible for several ailments, such as ventilator-associated pneumonia, bacteremia, urinary tract infections, and wound infections [128–130]. Currently, more than 60% of *A. baumannii* clinical isolates are multidrug-resistant (MDR), making the treatment of *A. baumannii* mediated infections increasingly problematic. Because of the shortage of effective treatments, The Centers for Disease Control and Prevention identified carbapenem-resistant *A. baumannii* as one of the most serious public health threats in 2019, and the World Health Organization prioritized the pathogen as critical for new antibiotic development, emphasizing its clinical significance[2,131–133]. This is a major concern because antibiotic treatment has been linked to the development of antibiotic resistance[134]. However, the mechanism by which *A. baumannii* adapts to and tolerates unfavorable environments, such as antibiotic treatments, remains unclear.

Investigating regulatory mechanisms that *A. baumannii* leverages to adapt to stress will help us to understand its pathophysiology and may lead to innovative treatment options. Transcriptome studies carried out with different *A. baumannii* strains, the *paa* operon has been identified as one of the most differentially regulated pathways when responding to various environmental challenges like antimicrobial treatment[21,104–107]. Despite the fact that metabolic pathways are routinely regulated as they react to environmental changes, Acinetobacter's PAA pathway is the sole amino acid catabolic system that is repeatedly and significantly controlled under a wide range of environmental variables[21]. The 13 genes that make up the *paa* operon (*paaABCDEFGHJKXYI)* encode enzymes that break down phenylacetic acid (PAA), a byproduct of

the amino acid L-phenylalanine's catabolism[21]. PAA catabolism involves several phases, including the formation of PAA-coenzyme A (PAA-CoA), followed by a distinct epoxidation phase that is dependent on *paaABCDE* [21]. The end product of PAA degradation is succinyl-CoA and acetyl-CoA which, under aerobic circumstances, enter the tricarboxylic acid (TCA) cycle[135,136]. Importantly, research has linked the *paa* operon to the pathogenicity of several Gram-negative bacteria, including *Burkholderia cenocepacia* and *A. baumannii*[109–112]. The pathogenicity of *B. cenocepacia* is decreased in a *Caenorhabditis elegans* model, for instance, when the upper PAA catabolic pathway (*paaABCDE*) is inactivated[109–112]. It was claimed that the decreased pathogenicity of the △*paa*A strain in zebrafish was due to greater quantities of cellular PAA, which functions as a neutrophil chemoattractant[110]. It is unknown how the PAA-dependent signaling cascade interacts with global regulatory networks *in A. baumannii*.

The sigma factor RpoS, which controls the transcription of multiple stress-related genes, orchestrates this response in the majority of Gammaproteobacteria[137]. Notably, *A. baumannii* lacks an RpoS homolog[138], suggesting that another system or system(s) regulates its general stress response. Transcriptional analysis reported that the virulence-associated two-component system (TCS) GacSA regulates the *paa* operon in *A. baumanni*[110,111]. When compared to the WT, the *A. baumannii gacS* mutant displayed more than 100-fold suppression of *paa* genes, , implying that GacS is a key regulator of the *paa* operon and may thus be responsible for regulating *paa* genes in response to changes in the surroundings[111]. We did not observe the same level of downregulation of *paa* operon genes when the cognate response regulator *gacA* was removed. Unlike many two-component systems, GacSA is not a continuous operon. GacA is an orphan response regulator which raises the possibility that it is involved in cross-signaling through another two-component system.

Two-component regulatory systems are found in both prokaryotes and plants and are utilized to sense and respond to specific signals, allowing adaptation to changing environmental conditions [139]. These systems are typically composed of a membrane-associated sensor kinase and response regulator [140]. The sensor kinase autophosphorylates a conserved histidine residue using an ATP molecule upon sensing an environmental signal[140]. Subsequent phosphotransfer from the sensor kinase to a conserved aspartate residue on the response regulator, induces a

conformational shift that often induces DNA binding to alter gene expression[140]. In a previous study, our group found that genes encoding the BaeSR TCS were induced as an adaptive response when *A. baumannii* shuts down biosynthesis of lipooligosacharides (LOS)[20]. The BaeS activating signals are not well-understood, but BaeS is thought to respond to elevated sucrose levels, suggesting it responds to changes in osmotic pressure [86].

In this study, we observed that the *paa* operon is regulated by the response regulator BaeR in addition to GacSA TCS, as the deletion mutant of *baeR* showed downregulation of the *paa* operon. Over expression of *baeR* can restored *paa* gene expression in Δ*baeR* and Δ*gacS* mutants. Sub-inhibitory concentration of kanamycin or gentamicin significantly altered growth of the Δ*baeR* mutant relative to wild type, demonstrating that Δ*baeR A. buamannii* cannot adequately respond to aminoglycoside-induced stress. In line with earlier research, we found that the *paaB* mutant, which controls cellular PAA levels, is vulnerable to oxidative stress, while the *baeR* mutant, which is a potential regulator of the *paa* operon, exhibits decreased survivability after exposure to $H_2O_2$[141]. We further showed that BaeR could control the expression of the *paa* operon by directly binding to the promoter and initiating transcription. Together, these findings offer new insights into how *A. baumannii* responds to stress.

**Materials and Methods**

Bacterial strains and growth conditions

*A. baumannii* strains ATCC 17978 and ATCC 19606 were cultured aerobically from freezer stocks on Luria-Bertani (LB) agar at 37°C. Unless otherwise specified, antibiotics were used at the following concentrations: kanamycin 25 mg/L, tetracycline 10 mg/L.

Construction of genetic mutants and complemented strains

*A. baumannii baeR, gacS, gacA*, and *paaB* mutants were constructed using the recombination-mediated genetic engineering (recombineering) technique, as previously described [142,143]. Using primers containing 125-bp flanking homology areas to the gene of interest, a kanamycin resistance cassette flanked by FLP recombination target (FRT) sites was PCR amplified from the pKD4 plasmid. The linear PCR product was then electroporated into *A. baumannii* strain ATCC 17978/pREC$_{Ab}$. Transformants were isolated in Luria broth and plated on LB agar containing 7.5 mg/L kanamycin. PCR validated all genetic mutations. After genetic mutants were isolated, the

pMMB67EH::REC$_{Ab}$ Tet$^r$ plasmid was eliminated as previously mentioned[142]. LB agar supplemented with 2 mM nickel (II) chloride (NiCl2) was used to cultivate isolated mutants before replica plating onto LB agar treated with kanamycin or tetracycline. PCR was used to demonstrate that mutants that were resistant to kanamycin and susceptible to tetracycline had lost the pMMB67EH::REC$_{Ab}$ Tet$^r$ plasmid. Cure mutants were transformed with pMMB67EH carrying the FLP recombinase (pAT08) and plated onto LB agar supplemented with tetracycline and 2 mM isopropyl b-D-1-thiogalactopyranoside (IPTG) to induce the expression of FLP recombinase to excise the chromosomal insertion of the kanamycin resistance cassette. PCR was used to verify that the kanamycin resistance cassette had been successfully removed. The BaeR complementation vector was created by amplifying the BaeR (A1S_2883) coding sequence (encoding BaeR) from *A. baumannii* ATCC 17978 genomic DNA (gDNA) and cloning it into the pMMB67EHknR plasmid's KpnI and SalI restriction sites. The generated pBaeR plasmids were transformed into the appropriate mutants and supplemented with 2 mM IPTG.

RNA sequencing

Transcriptome sequencing analysis was performed as described previously, with modification [20]. Briefly, the Direct-Zol RNA miniprep kit (Zymo Research) was used to extract total RNA from *A. baumannii* ATCC 17978, △*baeR,* △*gacS,* △*gacA,* WTpBaeR*,* △*baeR/*pBaeR, △*gacS/*pBaeR, △*gacA/*pBaeR. Strains were diluted 1:1,000 into 5 mL LB and grown to mid-exponential phase (OD600 of 0.4 to 0.6). Cultures were then treated with 2 mM IPTG for 30 min before RNA extraction. Isolated RNA was sent to the Microbial Genome Sequencing Center (MiGS) for Illumina NextSeq 550 sequencing. CLC genomic workbench software (Qiagen) was used to align the resulting sequencing data to the *A. baumannii* ATCC 17978 genome annotations and determine the reads per kilobase per million (RPKM) expression values and the weighted-proportions fold changes in expression values between meropenem-treated and untreated samples. Baggerley's test on proportions was used to generate a false discovery rate-adjusted P value. The weighted-proportions fold change in expression values between samples was used to generate pathway-specific heat maps in Prism 9.

qRT-PCR

RNA was extracted same way as RNA extracted for RNA sequencing. A Turbo DNA-free DNA removal kit (Invitrogen) was used to remove genomic DNA contamination. Following incubation, cells were harvested via centrifugation and resuspended in 500 µl RNA later and stored at -80˚C prior to RNA extraction. Relative-abundance quantitative PCR (qPCR) was performed as previously described[20,144]. In brief, the Sybr Fast One-Step qRT-PCR kit (Kapa Biosystems) was used with 16S rDNA as the internal reference. The PCR was performed using the Bio-Rad CFX Connect Real-Time PCR System. Relative expression levels were calculated using the ΔΔCt method[145], with normalization of gene targets to 16S rDNA signa

Biofilm assays

Biofilm were analyzed using a previously described method with slight modifications[146]. Overnight cultures were normalized to an $OD_{600}$ of 0.1 in polystyrene tube containing 1.5 ml Mueller–Hinton broth incubated for 48 h under static conditions at 37°C. After incubation, culture was washed twice with phosphate buffer saline (PBS) and stained with 2 mL 0.1% crystal violet solution dissolved in water for 15 min. The stain was eluted using ethanol: acetone (1:5) solvent. Absorbance of the eluted solvent was measured, after diluting 10-fold with the solvent, at 580 nm using an UV visible spectrophotometer.

Growth assays using antibiotics

Growth curves were performed in sterile, 96-well polystyrene plates with a circular bottom. In fresh medium (LB), bacterial strains were cultivated overnight in LB, washed in PBS, and diluted to an $OD_{600}$ of 0.01. Then, after being inoculated into 96-well plates with a final capacity of 150 mL, bacterial suspensions were cultured at 37 °C under shaking conditions. A BioTek microplate reader was used to measure $OD_{600}$ values throughout the course of 16 hours at 30-min intervals. The same procedure was followed to prepare bacteria in LB with the appropriate antibiotic concentration for antibiotic growth curves. At least three wells per strain and condition were used in each experiment, which was run over three independent days.

Protein purification

BL21 cells containing carrying pT7Kn::BaeR, were grown in 2L Luria broth and 1 mM IPTG at $37^0$C for 6h. Cells were collected and washed in cold 1x Phosphate-buffered saline (PBS), pelleted and the supernatant was removed. The dry pellet was frozen at -$80^0$C overnight. The pellet was thawed on ice and resuspended in 20 mL lysis buffer (20 mM Tris, 300 mM NaCl, 10 mM imidazole; pH 8). Cells were lysed using emulsiflex. To ensure proper lysis of cells the pressure is adjusted to 15-18k psi. Finally, lysed cell was incubated with lysis buffer washed His Pur Ni-NTA Resin (Thermo Scientific) on a rotator for 2 h at $4^0$C. Sample was added to a 10 mL protein purification column containing a porous polyethylene disk (Thermo Scientific) and allowed to gravity drip. The column was washed 3x with 10 mL lysis buffer and increasing concentrations of additional imidazole at each wash (0 mM, 15 mM, and 30 mM). 500 µl of elution buffer (20 mM Tris, 300 mM NaCl, 250 mM imidazole; pH 8) was incubated with the column for 5 min then gravity eluted 9 times. The elution fractions containing protein, as determined by a protein gel, were injected into a 10 mW dialysis cassette (Thermo Scientific) and dialyzed over-night in dialysis buffer (10 mM Tris, 50 mM KCl, 0.1 mM EDTA, 5% glycerol; pH 8) at $4^0$C. Purified protein was collected and verified using western blot with an anti-his antibody.

Electrophoretic Mobility Shift Assays (EMSA)

6XHis-BaeR proteins were purified as described above. EMSAs were performed based on a modified protocol[147]. A 344-bp DNA fragment of *paaA* spanning −265 to +79 relative to the transcriptional start site, and a 350-bp DNA fragment of *gyrA* flanking 300 bp upstream and 50 bp downstream of the start codon, were amplified. After BaeS-mediated phosphorylation of BaeR proteins[148], 0.1–1µM of BaeR proteins were incubated with $^{32}$P-labeled DNA at 25 °C for 20 min. For competition experiments, unlabeled P*paaA* and P*gyrA* were added at 1:1, 5:1, or 10:1 ratio relative to $^{32}$P-labeled P*paaA*, and1µM of phosphorylated BaeR proteins were used. After electrophoresis, gels were analyzed with a Storm 820 phosphor-imager according to manufacturer's instructions (Amersham Biosciences).

## Results

### Transcriptomic analysis indicates BaeR regulates Phenyl acetic acid (PAA) catabolic pathway

The PAA catabolic pathway, which is encoded by the *paa* operon, is a crucial step in the catabolism of numerous aromatic chemicals that converge and are directed to the Krebs cycle. Transcriptomic studies previously shown that *A. baumannii* 17978 responds to TMP/SMX therapy by upregulating the *paa* operon, which encodes the enzymes involved in phenylalanine catabolism, specifically the steps downstream of the intermediate PAA. Bacteria that metabolize phenylacetate were discovered more than 50 years ago[149] but the breakdown process wasn't fully understood until recently [136]. Numerous bacterial species have a role in the removal of environmental pollutants thanks to their capacity to metabolize aromatic compounds. The earliest metabolic pathway intermediates in infections are hypothesized to be involved in virulence[136]. Deletion of *baeR* resulted in repression of the entire *paa* operon (A1S_1336 – A1S_1349). Over expression of BaeR also upregulates the expression of *paa* operon genes both in *A. baumannii* 17978 and *A. baumannii* 19606 **(Figure 9a)**. Additionally, BaeR regulates numerous pathways as we have noticed downregulation of efflux pump genes, lipoproteins and *csu* operon **(Figure 9b)**. This is interesting as BaeR could potentially a stress response pathway gets activated with OM stress.

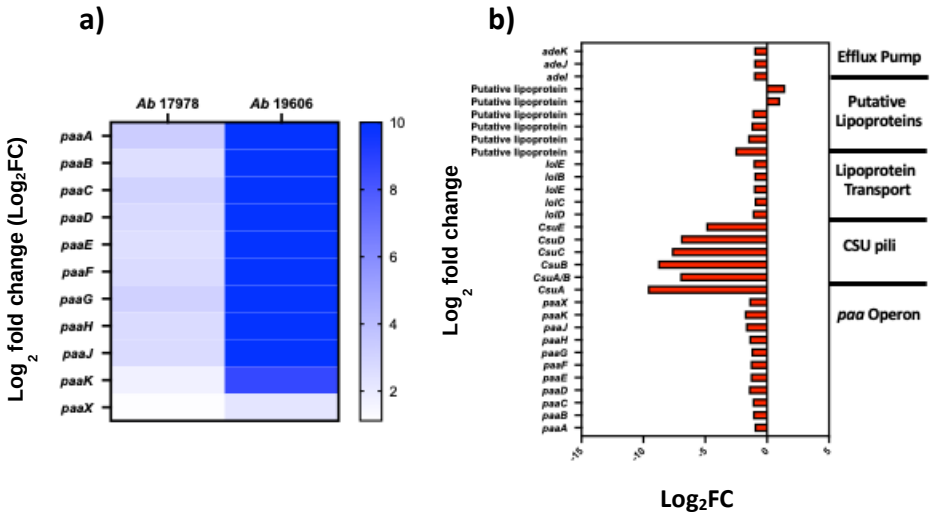



**Figure 9: Overexpression of BaeR upregulates Phenyl Alanine Metabolism, a) Heat map illustrating upregulation of genes in *paa* operon of *A. baumannii* 19606 and *A. baumannii* 17978 when BaeR is overexpressed, b) Bar diagram depicting the $Log_2FC$ of different pathway genes downregulated in △*baeR* compared to the WT.**

**GacS crosstalk with BaeR to control *paa* operon**

Previously it's been reported that GacS regulates the *A. baumannii* phenylacetate catabolic pathway [111]. GacS interacts with a response regulator known as GacA in other Gram-negative pathogens [150]. However, unlike PmrAB, AdeRS, and BaeSR, GacSA is not in a contiguous operon which suggests GacS is an orphan response regulator and may be involved in a cross-signaling through another two-component system. Since, we have established that BaeR, a response regulator of two component system BaeSR also regulates the PAA catabolism, we are curious to see whether complementation of △*gacS* with BaeR can rescue that expression of the *paa* operon. Interesting GacS and BaeR shared some pathways that are downregulated when we genetically mutate these proteins **(Figure 10b)**. In our transcriptomic analysis, we have seen that BaeR can rescue the repression of *paa* operon in △*gacS* which further supports the notion that BaeR co-regulates this PAA catabolism **(Figure 10c & 10d)**. qRT-PCR also confirmed that BaeR regulates the *paa* operon in △*gacS*.

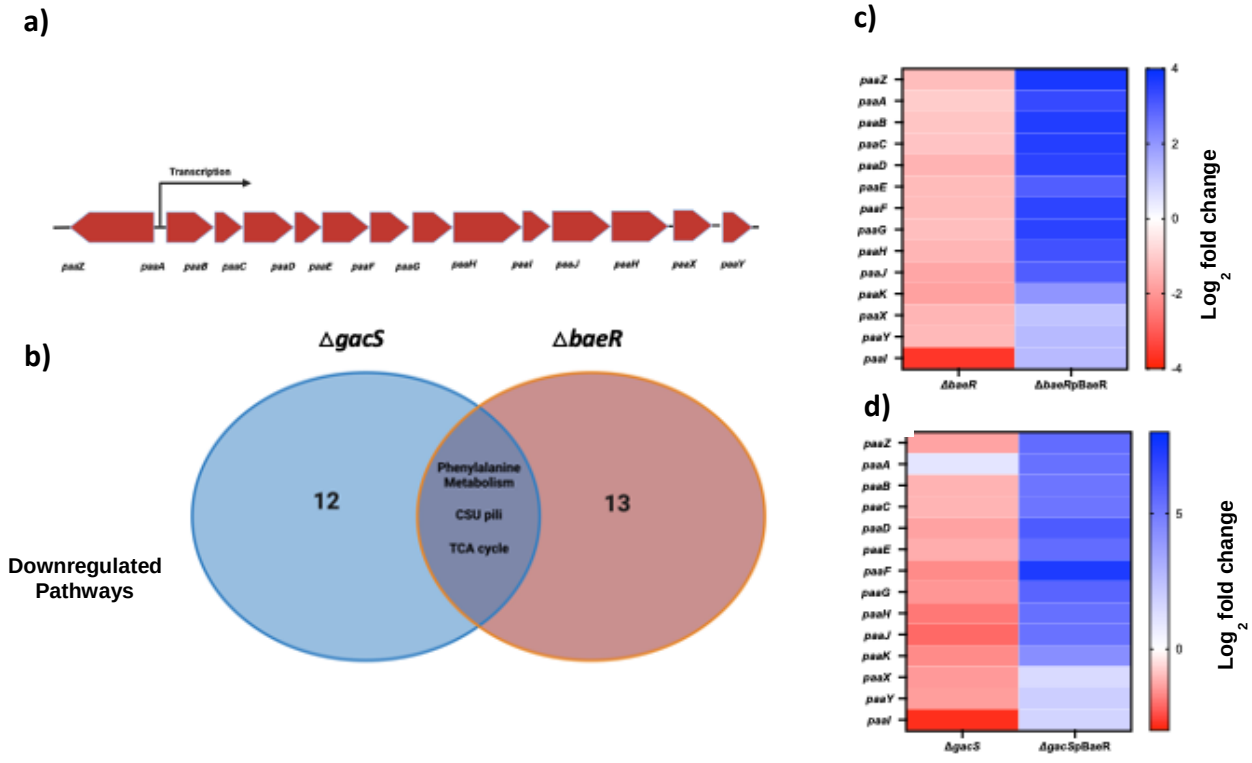



**Figure 10: GacS and BaeR coregulate *paa* operon, a) Schematic representation of *paa* operon, b) Ven-diagram depicting the downregulated pathways regulated by both GacS and BaeR, c & d) Heat map illustrating the fold change of *paa* operon genes when pBaeR is comlemented on △*baeR and* △*gacS***

**BaeR also regulates *csu* operon**

Biofilm formation is another way *A. baumannii* respond to stress. *csu* genes mediate the biofilm formation in *A. baumannii*. Both △*baeR* and △*gacS* transcriptionally regulated *csu* operon genes **(Figure 11b)**. We investigated their biofilm formation using crystal violate assay and the quantification indicates △*baeR* significantly produce less biofilm than wild type **(Figure 11c)**. Overexpression of pBaeR can restore the biofilm phenotype.

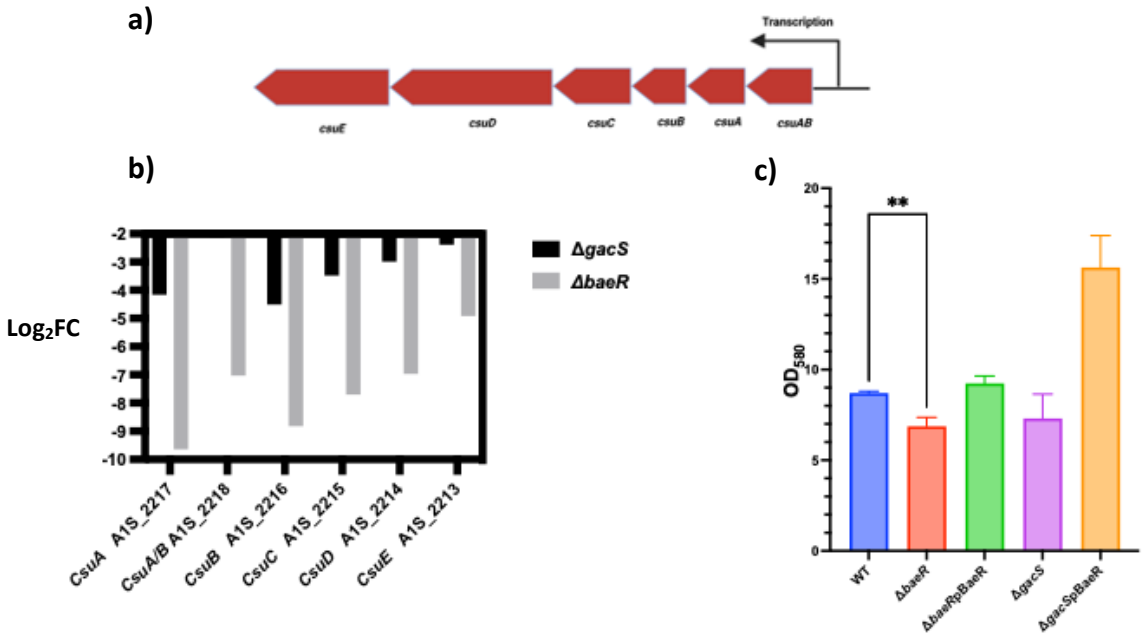



**Figure 11: Sensor Kinase GacS and Response Regulator BaeR also regulate Biofilm formation, a) Schematic representation of *csu* operon b) Bar digram showing the $log_2$FC in △*baeR* and △*gacS,* c) Crystal violet assay to quantify Biofilm formation in △*baeR* and △*gacS.***

**△*baeR* failed to respond against Aminoglycosidases**

Sub-MICs of kanamycin or gentamicin inhibited growth significantly in the *paaB* mutant relative to wild type, demonstrating that the *paaB* mutant strain cannot adequately respond to aminoglycoside-induced stress. Since BaeR regulates *paa* gene expression, we tested if △*baeR* growth would be defective when treated with antibiotics. In fact, △*baeR* showed delayed entry into growth phase when exposed to the antibiotic challenge **(Figure 12)**.

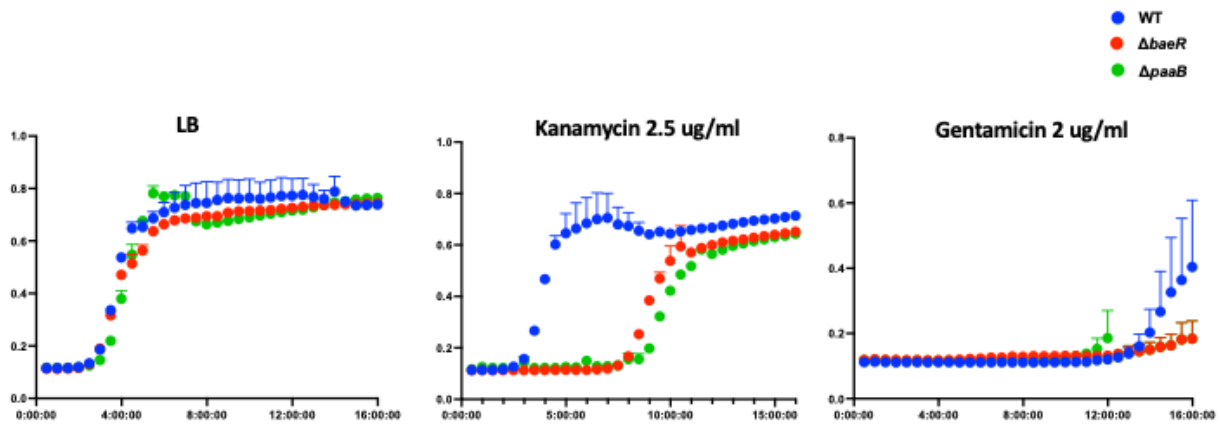



**Figure 12: △*baeR* failed to respond against Aminoglycosides. Representative growth curves of the WT and △*baeR*, △*paaB* strains in LB, and subinhibitory concentrations of kanamycin (2.5 mg/mL), and gentamicin (2 mg/mL).**

### BaeR$_{Ab}$ directly binds to PAA$_{Ab}$ Promoter

To determine if BaeR directly regulates *paa* gene expression BaeR$_{6x\text{-}His}$ was purified recombinantly and used for EMSA assays. In the presence of radiolabeled *paa* promoter and increasing BaeR$_{6x\text{-}His}$ concentrations, there, was an obvious shift in the *paa* promoter mobility, strongly suggesting a direct interaction (Figure 13). When unlabeled *paa* promoter was added in increasing concentration the shift was reduced, whereas competition with a nonspecific gyrA promoter, did not. In contrast, BaeR$_{6x\text{-}His}$ incubation with the *csu* promoter did not show a shift. together, these data shows that BaeR directly binds with the promoter of *paa* promoter but not the *csu* promoter.

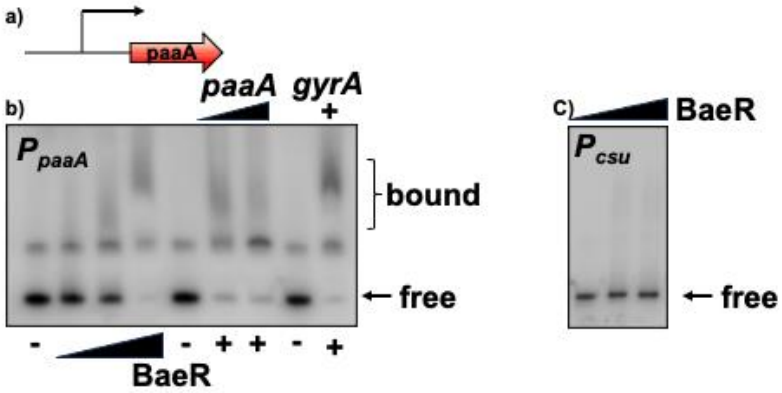



**Figure 13: Analysis of BaeR/DNA interactions for gene expression. (*A*) The *paaA* gene is the first gene in the *paa* operon. (*B* and *C*) EMSAs to analyze $^{32}$P-labelled DNA binding by BaeR proteins. (*B*) BaeR was used at 0, 0.1, 0.5, and 1 µM ) Lanes 1-4). Increasing ratios of unlabeled $P_{paaA}$ DNA (lane 6 - 1:1, or lane 7 - 10:1) or $P_{gyrA}$ DNA (lane 9 - 10:1) to labeled $P_{paaA}$ DNA were incubated with 1 µM of BaeR proteins. (*C*) BaeR was used at 0, 0.5, and 1 µM.**

**Discussion**

While *A. baumannii* strains may persist in the absence of lipid A production and acquire resistance to colistin and other clinically significant antimicrobials, we are still unsure of the cellular mechanisms or regulatory elements that let LOS deficient *A. baumannii* survive[19]. The deficiency of LOS results in different physical and chemical characteristics of the gram-negative cell envelope that affects membrane biogenesis and physiology and various -omics approaches were introduced to unveil these changes all together[78]. *A. baumannii* encodes Psp, Bae, and Cpx that react to stress or membrane disruptions by changing the cell envelope[151]. Therefore, learning more about the mechanisms that *A. baumannii* uses to adapt to stress will help us comprehend its pathophysiology and might result in the development of brand-new therapeutics. A global transcriptome investigation comparing various LOS$^-$ *A. baumannii* strains to their parent strains revealed that genes encoding the BaeSR two-component system (TCS) were upregulated in LOS$^-$ strains[20]. TCSs are common signal transduction systems in bacteria[152], and five two-component systems have been discovered in *Acinetobacter*, including BaeSR[89]. Furthermore, whereas *baeSR* genetic deletion was not required for wild type *A. baumannii* life, LOS- *A. baumannii* could not be restored, indicating that BaeSR controls some important function that enables LOS survival.

Interestingly, Phenyl alanine catabolism genes are highly upregulated when pBaeR is overexpressed. We utilized RNA-seq to identify BaeSR-dependent regulatory products in wild type *A. baumannii* strain ATCC 19606, △*baeR*, and △*baeR*pBaeR, WTpBaeR where BaeR expression is controlled by an inducible promoter. We and others have demonstrated that the *paa* operon, which is responsible for PAA degradation, one of the most differently regulated pathways in response to stress, and is highly elevated under antibiotic therapy. Because of elevated quantities of released PAA, the △*paa* demonstrated decreased virulence in the zebrafish model and served as a neutrophil chemoattractant. It's been reported that GacSA TCS regulates *paa* operon genes but both of the sensor kinase, GacS and response regulator GacA located far apart in the genome. This likely speculate that the GacS may communicate with different response regulator to exhibit its function. According to the gel shift assay BaeR is directly bound to the promoter of *paa* operon confirmed the direct regulation **(Figure 14)**. *csu* operon

genes responsible for biofilm formation are also upregulated with pBaeR overexpression. However, of the *csu* promoter did not directly bind to BaeR, so regulation appeared to be indirect. *csu* expression is well known to be controlled by the BfmRS TCS[153]. This is the first report of BaeR regulated stress response in *A. baumannii*.

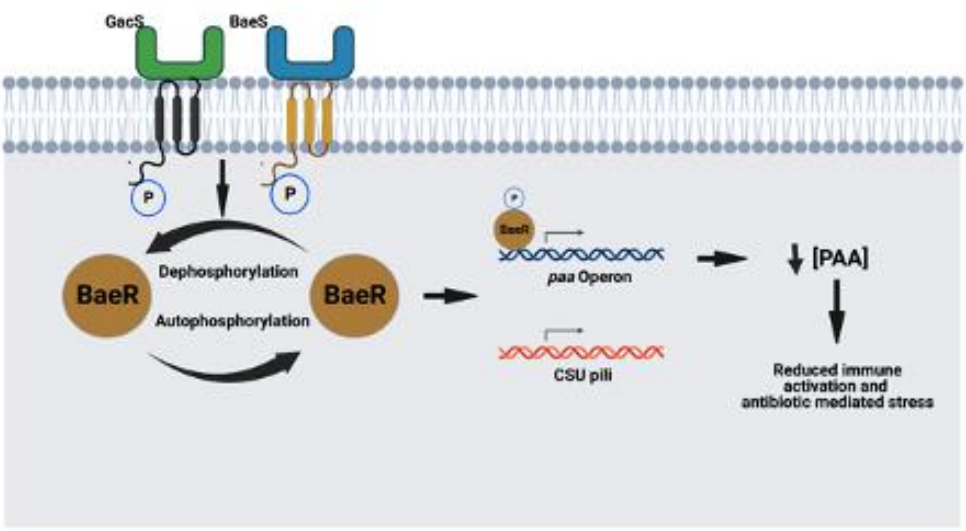



**Figure 14: Proposed model of signaling pathway regulated by BaeR. Upon sensing the perturbation in OM BaeR activates its signaling cascade by directly binding to the promoter of *paa* operon and indirectly regulate *csu* operon.**

## CHAPTER 4

## Antimicrobial activity against *A. baumannii* using ruthenium (RU) (II) based photosensitizers


### Abstract

Antimicrobial resistance poses a severe threat to public health. Many pathogens can survive and even thrive in the presence of antimicrobial agents due to resistance mechanisms. Most research to develop new antimicrobials relies on derivatizing FDA-approved compounds to increase their efficacy. However, an abundance of research suggests that bacteria will always finda way to overcome antimicrobial treatment and survive, eventually rendering such compounds useless. Conventional strategies relying on one medicine to treat one target has proven unsustainable; however, an alternate strategy that involves perturbing multiple vital pathways is promising. Although photo-antimicrobials have been available to treat cancer cells, their utility as antibacterial agents have not been adequately explored. The use of photo-antimicrobials is superior to traditional antimicrobial agents because they perturb multiple bacterial targets, making them less likely to select for resistant variants. We created a Ruthenium (RU)-based photosensitizer that is now in phase II clinical trials as an anticancer drug, and we wanted to measure the antimicrobial activity of the compound and closely related derivatives against difficult-to-kill Gram negative bacteria. Specifically, *A. baumannii* growth and survival was evaluated against RU-based photosensitizers and derivatives. In their activated form, the chemicals release reactive oxygen species (ROS). We also tested the chemical against MDR *A. baumannii* strains and determined the $EC_{50}$ and $EC_{90}$ values. Several compounds are effective against MDR *A. baumannii*, but $[Ru(5,5'\text{-dmb})_2(IP\text{-}1T)]Cl_2$ showed the lowest $EC_{90}$ and the highest phothterapeutic indices (PI). Our attempt to isolate resistant mutants failed suggesting that the levels of reactive oxygen species generated by the compounds were high, and presumably difficult to overcome. The CDC has designated *A. baummanii* to be an urgent threat, and herein we evaluate several photosensitizers with low mM activity against *A. baumannii* to evaluate their efficacy.

**Introduction**

A major public health issue today is antimicrobial resistance (AMR) because our current antibiotic arsenal is no longer effective against many pathogens[1,154]. By 2050, AMR is predicted to be responsible for more than 10 million fatalities annually[155]. Given the simplicity and effectiveness antibiotics when the microorganisms exhibit no resistance, a widespread assumption is that their effectiveness will last forever [118]. We now know this is not the case du to circulating resistance mechanisms. Furthermore, most antimicrobial agents that entered the market in recent years are derivatives of agents that already exist, and as a result, they lack a novel mode of action[156]. However, microbe replicate quickly to high numbers and rapidly evolve resistance to a wide range of expensive antimicrobial drugs[157]. A single mechanism of actions the primary reason that microbes quickly develop resistance[158]. Development of therapeutics with multiple mechanisms of action and targets is a superior strategy to prevent the resistance development[118]. Teixobactin, a prospective novel antibiotic, is intriguing in this regard because it can go after several targets in the face of resistance; nonetheless, it looks to be most effective against just Gram-positive bacteria[159]. Halogenated xanthenes (Rose Bengal), phenothiazines (Toluidine Blue, Methylene Blue), conjugated macrocycles like acridines, phthalocyanines, and porphyrins, as well as natural products like hypericin, have all been investigated for their potential to kill pathogenic bacteria[119]. While these neutral and anionic organic photosensitizers(PS) easily inactivate Gram-positive bacteria, it is still difficult to specifically target Gram-negative bacteria[160]

*Acinetobacter baumannii* is a Gram-negative bacterium that is responsible for causing a wide range of infections, particularly in healthcare settings such as hospitals where the most susceptible populations reside. It is considered an opportunistic pathogen, meaning it primarily affects individuals with compromised immune systems or those who are already ill. One reason *A. baumannii* is difficult to treat is its propensity to develop antibiotic resistance. Due to their potent broad-spectrum activity and resistance to typical resistance mechanisms, carbapenems are important beta-lactam therapeutics[161,162]. Meropenem is a carbapenem antibiotic used as a last resort to treat Gram-negative infections that are resistant to many drugs[8,9]. *A. baumannii* is primarily treated with meropenem, which is normally used to combat multidrug resistance

bacteria[10,11]. Hospital acquired infections now frequently contain *A. baumannii* that is carbapenem-resistant [163]. The carbapenem-resistant *A. baumannii* was cited by the Centers for Disease Control as one of the most serious dangers to public health in 2019[2]. More recently, the World Health Organization identified the pathogen as crucial for the development of new antibiotics, underlining the severity[3].

To overcome the notion of continuous development of antimicrobial resistance, a novel treatment option unlike one site of action model typical of conventional antibiotics appears potential. One such strategy is photodynamic inactivation (PDI), also known as photodynamic antimicrobial chemotherapy (PACT) or antimicrobial photodynamic therapy (aPDT), which is particularly distinctive in that it has not been demonstrated to be susceptible to antibiotic resistance[164–166]. The photodynamic antimicrobial concept is based on the combination of visible or near-infrared light, oxygen, and a photo-antimicrobial that can absorb and transfer energy or electrons to molecular oxygen after light absorption to produce reactive oxygen species[118]. **(Figure 15)** A wide range of molecular microbial targets, such as proteins, lipids, and nucleic acids, can be destroyed by reactive oxygen species, such as singlet oxygen, superoxide anions, and hydroxyl radicals[119,167]. Recently, it was shown that cationic PS, including certain Ru(II) complexes, have the ability to inactivate Gram-negative bacteria[168]. According to current thinking, electrostatic interactions with the negatively charged outer membrane of gram-negative bacteria, which preclude neutral and anionic PS, provide the cationic PS with improved binding. Increased membrane penetration of the cationic PS ensures that photogenerated singlet oxygen has the detrimental intracellular effect on the microorganism that has been attributed to it[160].

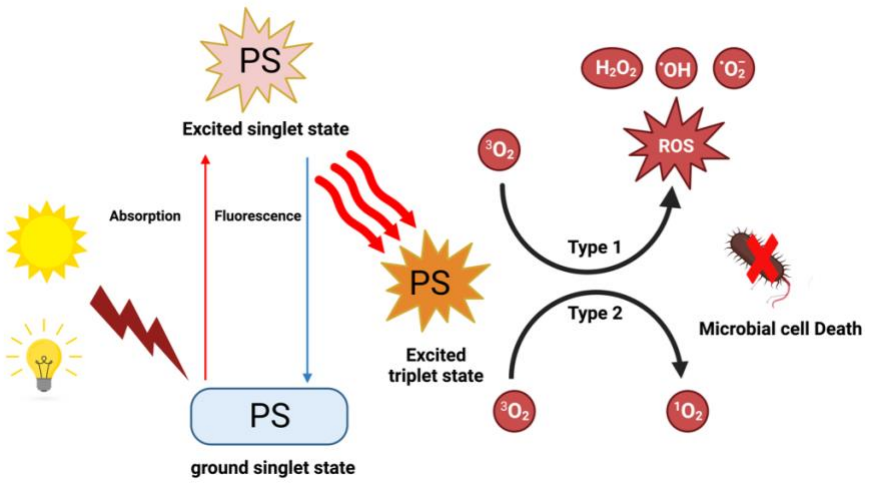



**Figure 15: Mechanism of photosensitizers action. Longer lived triplet state interact with Oxygen by two mechanisms. In type 1, generation of ROS. In type 2, the triplate state of the compound undergo energy exchange with triplet ground state oxygen, leading to the formation of excited oxygen.**

While antimicrobial PDT is still largely underappreciated by clinicians and organizations in charge of providing healthcare, anticancer PDT is now a clinical reality in hospitals and dermatology clinics all over the world, including for the treatment of actinic keratosis and basal cell carcinoma for more than 25 years[118,169]. We synthesized a compound, $[Ru(4,4'\text{- dmb})_2(IP\text{-}3T)](Cl)_2$ is in Phase II clinical trials for treating non-muscle invasive bladder cancer with light (ClinicalTrials.gov Identifier: NCT03945162) **(Figure 16)**. The phototherapeutic indices (PIs) as large as $10^{10}$ in certain cell lines when activated with visible light[170]. Some of these PSs maintain their activity even under hypoxia, a condition under which cancer cells are

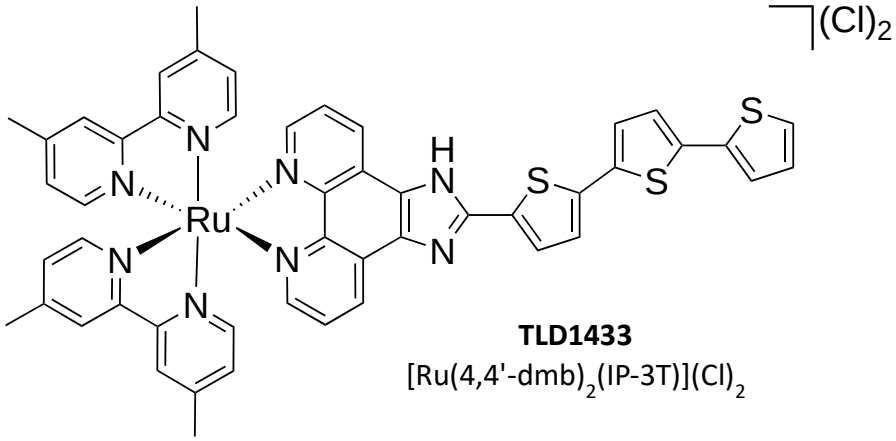



**Figure 16: Chemical structure of TLD1433**

(dmb: dimethyl bis-pyridine; IP: [4,5-f][1,10- phenanthroline]

notoriously difficult to destroy[22,171–173]. Although the mechanisms underlying this remarkable potency are unknown, PSs are known to be efficient ROS producers, therefore we attribute at least some of the activity in normoxia to lethal singlet oxygen. We developed this PSs as an alternate treatment to treat *A. baumannii* infections. Furthermore, derivatives of this compound now in clinical trials have the capacity to kill 90% ($EC_{90}$) of *A. baumannii* population at low micromolar concentrations. As a result, we feel this is a powerful alternative option for clinical care of a disease that desperately needs an antibiotic.

**Materials and Methods**

**Photosensitizers**

More than thirty PSs are used and are based on Ru(II) and Os(II). Powders and solutions for both compounds were kept at room temperature (RT) in the dark. Stock solutions were prepared in DMSO at a concentration of 5 mM unless otherwise noted. According to CLSI recommendations, [Ru(II)] were evaluated for concentrations of 64 M in each test, bringing the final concentrations of DMSO to levels that did not exceed 1%.

**Illumination Set-Up and Light Treatment Procedure**

The irradiance of the light source was measured (I; $W/cm^2$)) using the power meter and the time of illumination (t) was calculated using desired fluence (F; $J/cm^2$), using equation; F= I×t. If more lighting is necessary then it was made sure that the power output is comparable or, ideally, utilize the same brand and power-output source. The setup of light source illuminating the plates are in **Figure 3a**.

**Bacterial Strains & Cell Culture Conditions**

All strains were grown in lysogeny broth (LB) liquid medium at 37 °C with 200 rpm shaking, unless otherwise stated.

**Determination of MIC and MBC**

Overnight cultures were normalized to an $OD_{600}$ of 0.05 in LB and inoculated into sterile, round-bottom polystyrene 96-well plates (Corning, Inc.) with a final volume of 50µL per well. prepare Compound dilutions are prepped in pre-decided (standard; 300µM to $10^{-3}$µM or ultra-low; 300µM to$10^{-12}$µM) in a deep well plate using Milli-Q water. 50µL of compound dilutions to all the sample wells was added. The light plates were irradiated in a fluence of 100 $J/cm^2$ for required

amount of time and incubated for 20 hrs. The workflow is illustrated in **(Figure 17b)**. From 96 well plates 5µL of all the dilution was spotted in Lb agar plate to determine any possible colonies. Sports showing no growth was determined as zero cell viability in our activity plots and dose response curves.

**Evaluation of $EC_{50}$, $EC_{90}$ and PI values**

In order to determine the concentration of PS necessary to lower cell viability by 50% ($EC_{50}$ value) and 90% ($EC_{90}$ value) in both dark and light conditions, dose-response curves will be created using Graphpad Prism 9. To measure the activity of each PS, the PI will be computed as the ratio of dark to light $EC_{50}$ values.

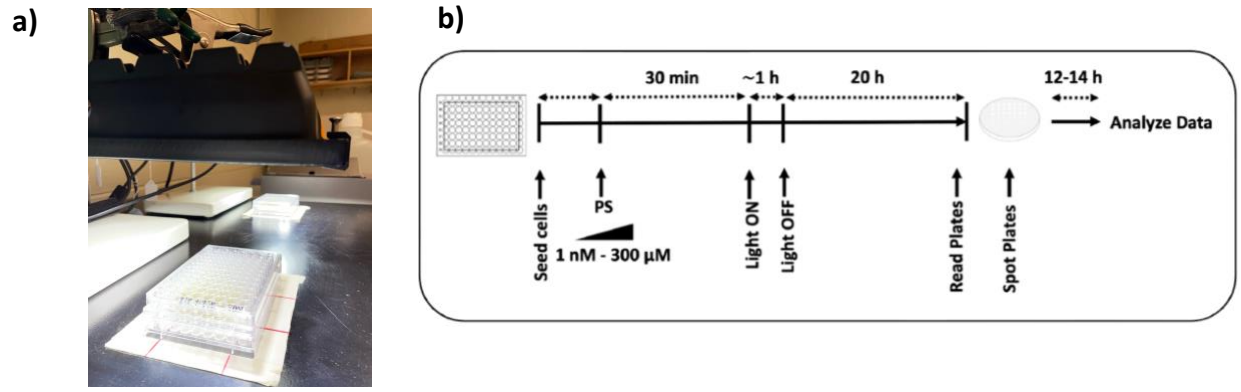



**Figure 17: MIC determination under light, a) Set-up of the illumination of plates under light. B) the workflow for MIC assay**

**Results**

We posited that these PSs would be beneficial against AMR bacteria considering the powerful activity seen for certain metallodrugs of this PS family against difficult-to-treat cancer cells and the generally nonspecific cellular lethality of ROS (Reactive oxygen species) and other RMS (Reactive molecular species). Numerous Ru(II) and Os(II) based photosensitizers (PSs) that we have developed exhibit impressive in vitro photocytotoxicity against a range of cancer cell lines, including melanoma as well as lung, breast, and prostate cancer. The reference compound **(Figure 18)** used here has four thiophene rings as its well established that three/ four thiophenes are potent against cancer cell. We evaluated the light-triggered activity of 15 distinct Ru and Os PSs (Table 1) against wild-type *A. baumannii*.

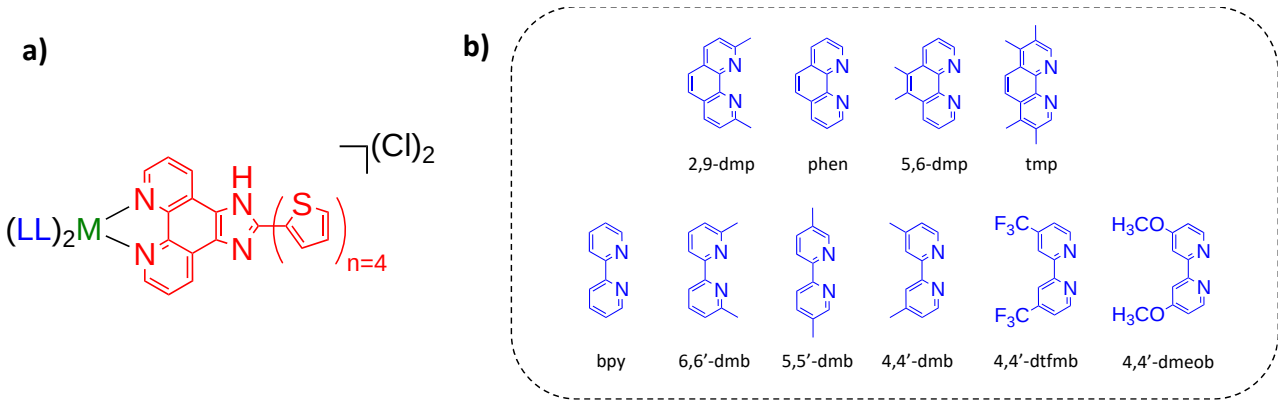



**Figure 18: PS scaffold selected for screening in *A. baumannii* a) $[M(LL)_3](Cl)_2$: Reference compound that lacks functional ligand(M= Ru(II), Os(II) LL=co-ligands, IP-nT=functional ligand for RMS generation) b) List of co-ligands**

Table 1: List of compounds Initially screened for their activity against *A. baumannii* 17978

| | | |
|---|---|---|
| 1: $[Ru(2,9\text{-dmp})_2(IP\text{-}4T)]Cl_2$ | 6: $[Ru(5,5'\text{-dmb})_2(IP\text{-}4T)]Cl_2$ | 11: $[Os(5,6\text{-dmp})_2(IP\text{-}4T)]Cl_2$ |
| 2: $[Ru(phen)_2(IP\text{-}4T)]Cl_2$ | 7: $[Ru(4,4'\text{-dmb})_2(IP\text{-}4T)]Cl_2$ | 12: $[Os(tmp)_2(IP\text{-}4T)]Cl_2$ |
| 3: $[Ru(5,6\text{-dmp})_2(IP\text{-}4T)]Cl_2$ | 8: $[Ru(4,4'\text{-dtfmb})_2(IP\text{-}4T)]Cl_2$ | 13: $[Os(bpy)_2(IP\text{-}4T)]Cl_2$ |
| 4: $[Ru(bpy)_2(IP\text{-}4T)]Cl_2$ | 9: $[Ru(4,4'\text{-dmeob})_2(IP\text{-}4T)]Cl_2$ | 14: $[Os(4,4'\text{-dmb})_2(IP\text{-}4T)]Cl_2$ |
| 5: $[Ru(6,6'\text{-dmb})_2(IP\text{-}4T)]Cl_2$ | 10: $[Os(phen)_2(IP\text{-}4T)]Cl_2$ | 15: $[Ru(4,4'\text{-dtfmb})_2(IP\text{-}3T)]Cl_2$ |

**PDI towards *A. baumannii***

We have tested both the killing and inhibitory activity of the PSs in the absence and presence of light activation. The 15 compounds were evaluated for growth inhibitory activity by sstandard minimum inhibitory concentration (MIC) assays (using an initial cell inoculum of $1x10^6$ CFU $mL^{-1}$)[174]. Interestingly, only one out of 15 PSs $[Ru(4,4'\text{-dtfmb})_2(IP\text{-}4T)]Cl_2$, was able to suppress the growth of *A. baumannii* **(Figure 19a & 19b)**. We calculated the MIC was approximately 25 µM upon visible light treatment with a fluence (the amount of light energy delivered per unit of area) of 100 J $cm^{-2}$ delivered at a rate of 21-23 mW $cm^{-2}$ **(Figure 19c)**. This activity is similar to that of meropenem, a known antibacterial drug with an MIC of 0.42 µM (0.16 µg $mL^{-1}$) against *A. baumannii*[133]. This compound is denoted as compound #8 in our order, and we will use this nomenclature in rest of this document.

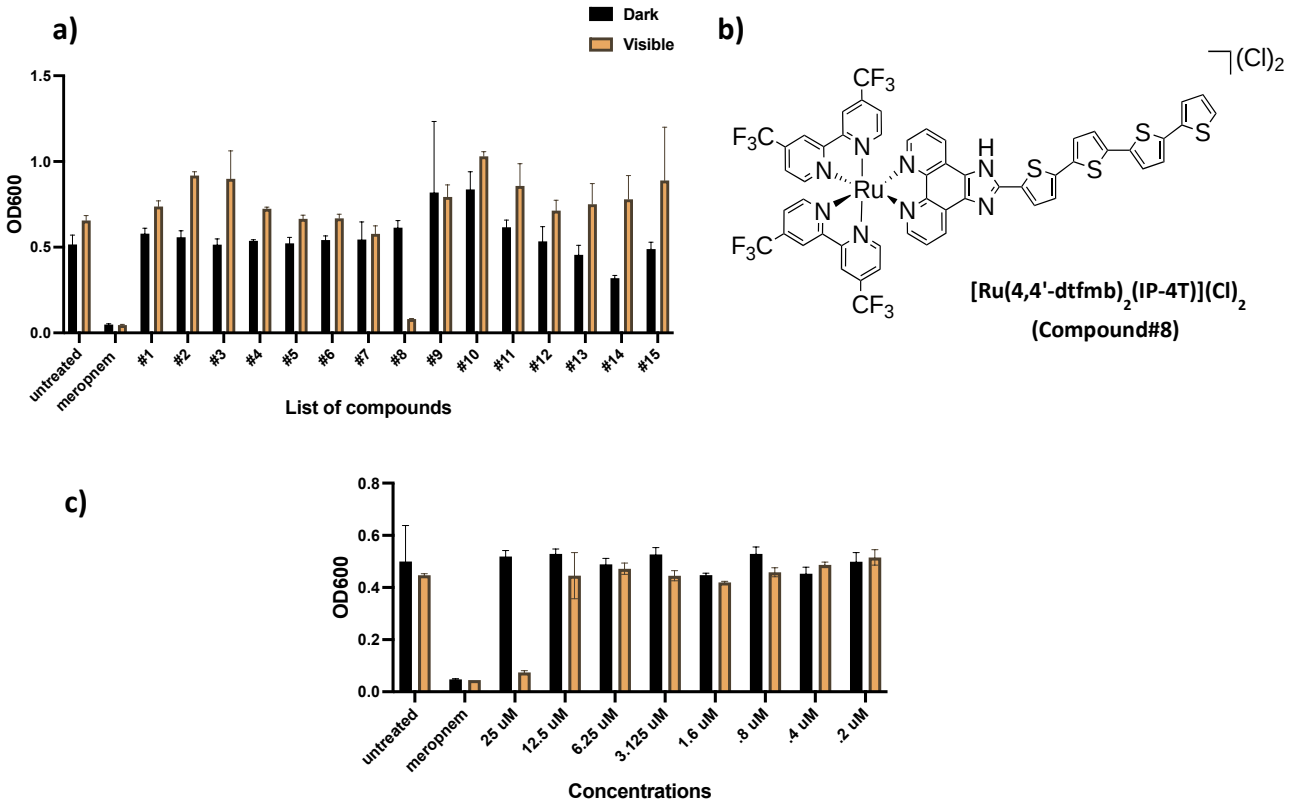



**Figure 19: Activity of *A. baumannii* of in normoxia a) Growth inhibition of *A. baumannii* against all fifteen compounds with three and four thiophenes. b) Chemical structure of compound #8 c) Determination of MIC of compound #8**

**Side chain with $CF_3$ is critical for the bacteriostatic activity**

Given that its nonfluorinated cousin 7 was fully ineffective against *A. baumannii*, this finding for compound 8 was unexpected. Like their Os counterparts (10-14), other Ru compounds with the IP-4T ligand that were coupled with various co-ligands (1-7, 9) were inactive. We are also testing new PSs for action against wild type *S. aureus* and *E. coli* as model Gram-positive and -negative bacteria in order to simplify the selection of compounds for testing against *A. baumannii*. To begin we compared a family of compounds based on the $[Ru(4,4'\text{-dtfmb})_2(IP\text{-nT})](Cl)_2$ scaffold that differ in the number of thiophene rings in the IP-nT ligand **(Figure 20a, 20b & 20c)**. These compounds are derivatives of compound #8 and we wanted to explore whether the no of thiophenes has any effect on their bacteriostatic ability. We also included $[Ru(4,4'\text{-dtfmb})_3](Cl)_2$ and $[Ru(4,4'\text{-dtfmb})_2(phen)](Cl)_2$ denoted as compound #20 and #21 as reference compounds that were expected to be inactive.

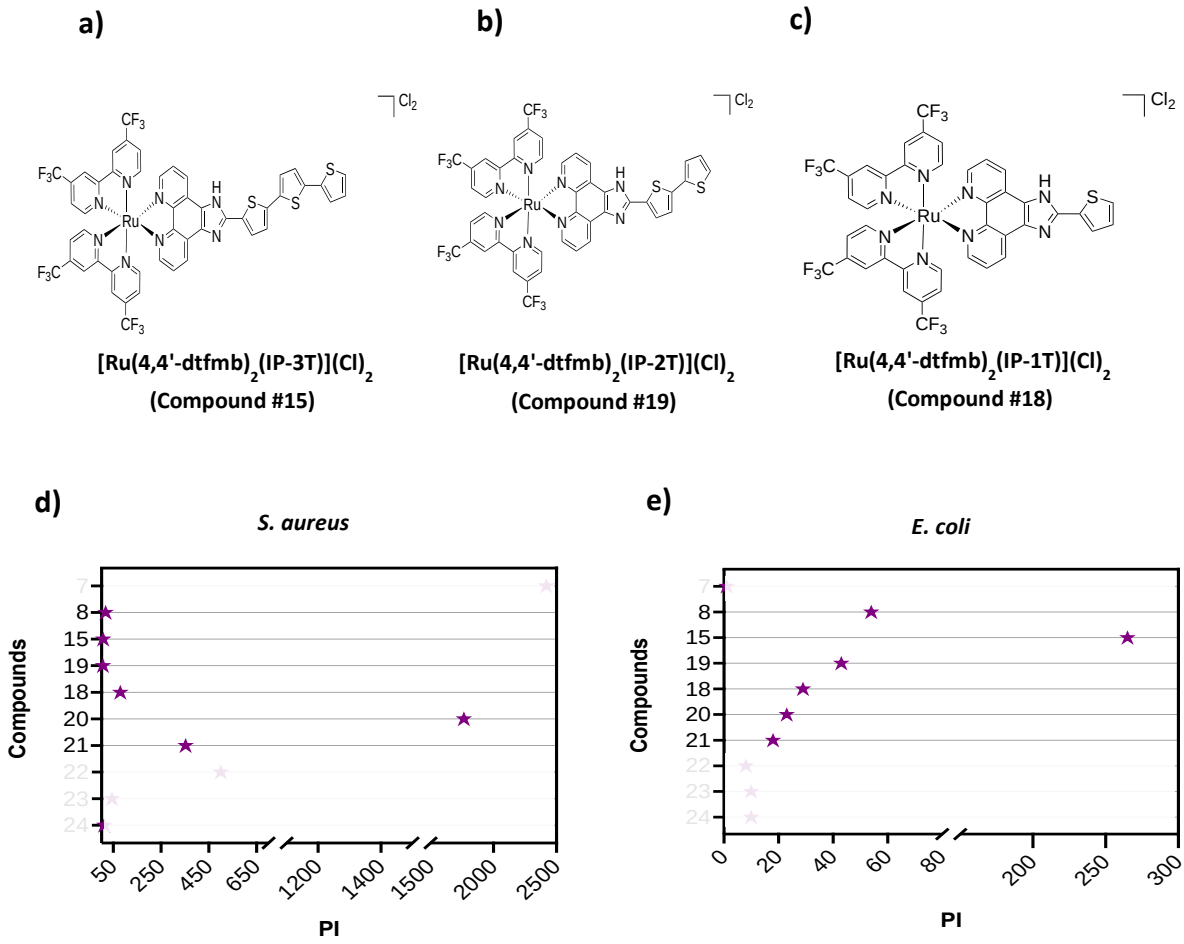



**Figure 20: Antimicrobial activity of PSs with fluorinated side chains; a & b & c) Chemical structure of fluorinated compounds 15, 19, 18; d & e) Activity plot of phototoxicity of selected compounds against *S. aureus* and *E. coli***

Unexpectedly, we discovered the opposite trend for *S. aureus* activity compared to what we saw in other cancer cell lines tested: Compound #20 was the most active (PI >1700), and compound #8 was the least active (PI=18) **(Figure 20d)**. The trend was reversed in *E. coli* and coincided with the pattern shown in cancer cells, which was even more striking **(Figure 20e)**. Compound #15 was the most active (PI=265) and compound #20 was the least active (PI=23). We decided to pursue compound #15 and compound #19 for next phase of experiments along with compound #8. Even though the nonfluorinated PSs were ineffective against *A. baumannii*, we evaluated the comparable family against *S. aureus* and *E. coli* to check if the pattern we saw for the fluorinated family against these two bacteria was consistent. Surprisingly, $[Ru(4,4'\text{-dmb})_2(IP\text{-}4T)](Cl)_2$ denoted as compound #7 **(Figure 21a)** was the most effective chemical against *S. aureus* (PI=>2400) but not against *E. coli* **(Figure 21b & 21c)**. This is the inverse of what we saw with

compound 8. These unexpected results raise the question of whether the activity is driven by the physicochemical or photophysical features of the PSs, or by a mix of both.

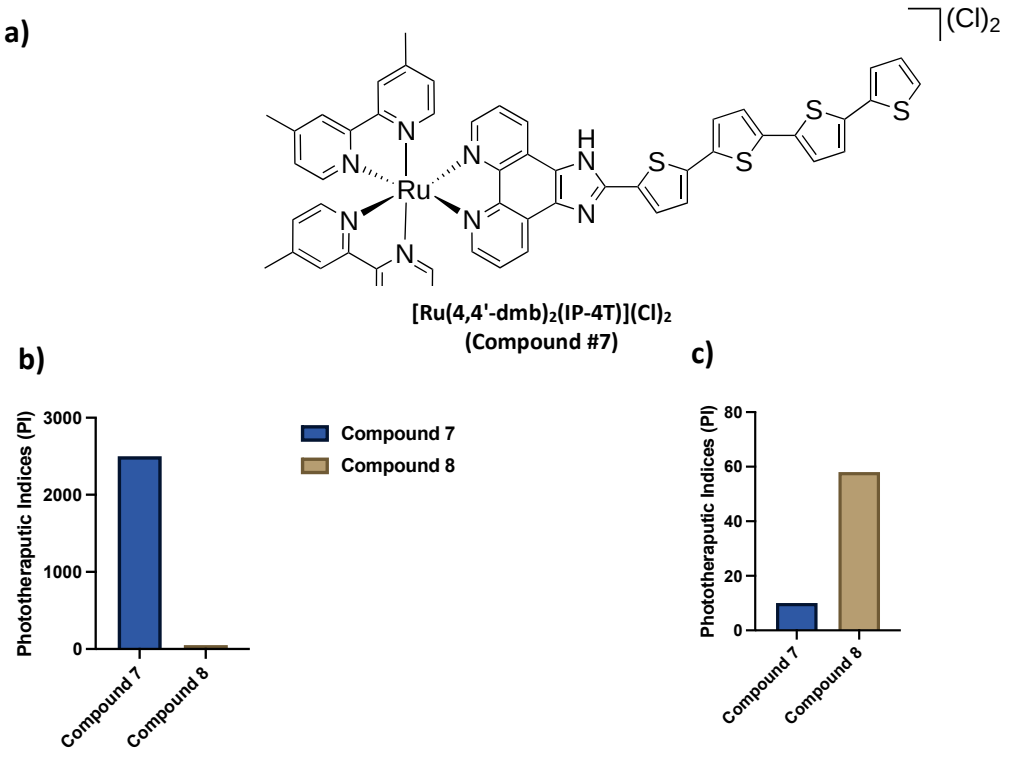



**Figure 21: Non-fluorinated side chains showing antimicrobial activity against *S. aureus* and *E. coli*; a) Chemical structure of compound #7; b & c) Phototherapeutic indices (PI) of compound #7 against *S. aureus* and *E. coli* respectively.**

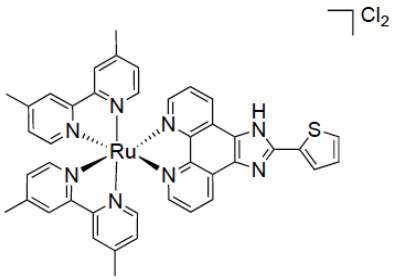
**No of thiophene rings do not determine the efficacy of photosensitizers**

We were curious to see whether no of thiophenes or the positioning of di-methyl group control the activity of these photosensitizers. A list of 7 compounds from each parent strains where the dimethyl group located at 4,4 position or 5,5 position are used. List of this compounds used to screen the activity against wild type *A. baumannii* are mentioned in Table 2. We determined the EC50 value in μm concentrations, and it came out that four of the fourteen compounds exhibited a lower EC50 against the *A. baumannii* population when illuminated by light. Interestingly, these four compounds either have one or two thiophen rings. It suggests that the lengthening of the thiophene ring will not necessarily increase the PSs' efficacy. These four drugs have been included to the next round of testing against the multidrug-resistant *A.*

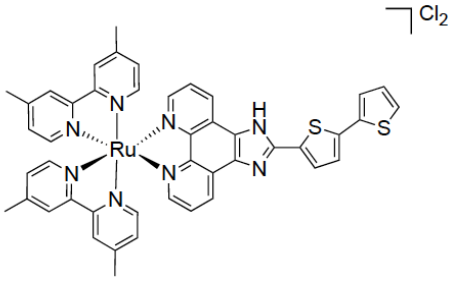
*baumannii* 5075 pathogen and other ESKAPE diseases. A selection of seven compounds **(Figure 22)** that were mainly selected for our next phase of investigation includes these four compounds along with the three compounds with dimethyl fluorine side chains stated before.

Table 2. Cytotoxicity and photocytotoxicity observed in *A. baumannii* 17978 cells under normoxia

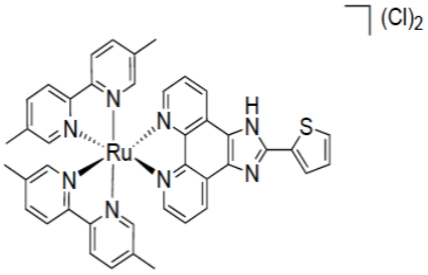
| **Complex** | **$OD_{600}$-Cell Viability** | | |
|---|---|---|---|
| | $EC_{50}$ ± SEM (μM) | | PI |
| | Dark | Visible | Visible |
| **$[Ru(4,4'\text{-}dmb)_3]Cl_2$** | >300 | >300 | 1 |
| **$[Ru(4,4'\text{-}dmb)_2(phen)]Cl_2$** | >300 | >300 | 1 |
| **$[Ru(4,4'\text{-}dmb)_2(IP\text{-}0T)]Cl_2$** | >300 | >300 | 1 |
| **$[Ru(4,4'\text{-}dmb)_2(IP\text{-}1T)]Cl_2$** | 143 ± n.d. | 1.90 ± n.d. | 75 |
| **$[Ru(4,4'\text{-}dmb)_2(IP\text{-}2T)]Cl_2$** | 145 ± 31 | 3.65 ± n.d. | 40 |
| **$[Ru(4,4'\text{-}dmb)_2(IP\text{-}3T)]Cl_2$** | >300 | 35.6 ± n.d. | >8 |
| **$[Ru(4,4'\text{-}dmb)_2(IP\text{-}4T)]Cl_2$** | >300 | >300 | 1 |
| **$[Ru(5,5'\text{-}dmb)_3]Cl_2$** | >300 | >300 | 1 |
| **$[Ru(5,5'\text{-}dmb)_2(phen)]Cl_2$** | >300 | >300 | 1 |
| **$[Ru(5,5'\text{-}dmb)_2(IP\text{-}0T)]Cl_2$** | >300 | >300 | 1 |
| **$[Ru(5,5'\text{-}dmb)_2(IP\text{-}1T)]Cl_2$** | >300 | 1.24 ± n.d. | 242 |
| **$[Ru(5,5'\text{-}dmb)_2(IP\text{-}2T)]Cl_2$** | 150 ± 98 | 1.67 ± n.d. | 90 |
| **$[Ru(5,5'\text{-}dmb)_2(IP\text{-}3T)]Cl_2$** | >300 | >300 | 1 |
| **$[Ru(5,5'\text{-}dmb)_2(IP\text{-}4T)]Cl_2$** | >300 | >300 | 1 |
| **Meropenem** | 0.167 ± 0.045 | 0.131 ± 0.026 | N/A |



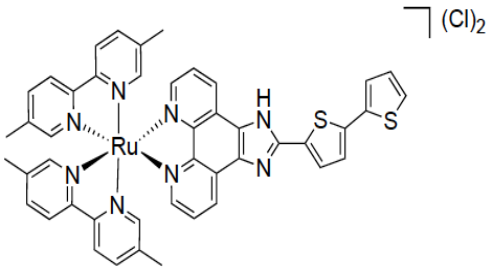
**[Ru(4,4′-dmb)$_2$(IP-1T)]Cl$_2$**



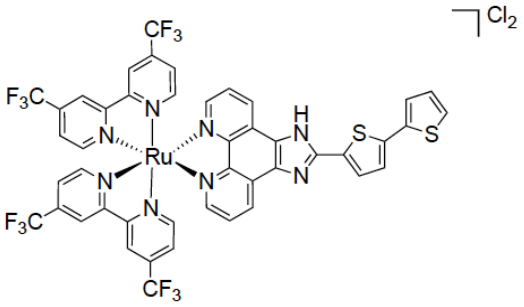
**[Ru(4,4′-dmb)$_2$(IP-2T)]Cl$_2$**



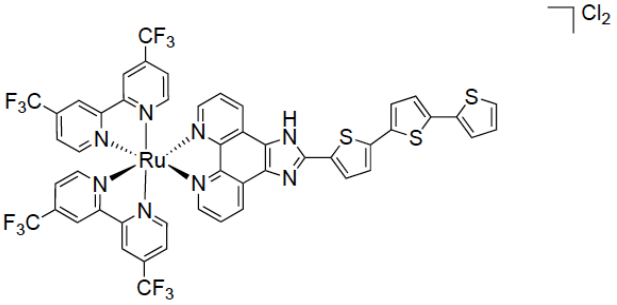
**[Ru(5,5′-dmb)$_2$(IP-1T)]Cl$_2$**



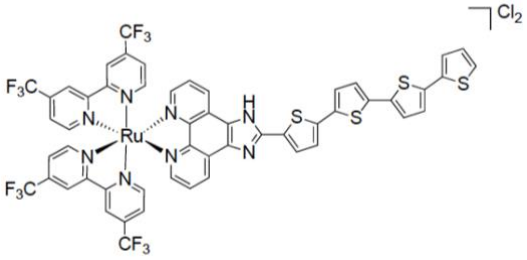
**[Ru(5,5′-dmb)$_2$(IP-2T)]Cl$_2$**



**[Ru(4,4′-dtfmb)$_2$(IP-2T)]Cl$_2$**



**[Ru(4,4′-dtfmb)$_2$(IP-3T)]Cl$_2$**



**[Ru(4,4′-dtfmb)$_2$(IP-4T)]Cl$_2$**

**Figure 22: Chemical structure of seven selected compounds for screening against MDR *A. baumannii***

**Determining $EC_{50}$ and $EC_{90}$ values of *A. baumannii* against seven selected compounds**

We evaluated the potency of the selected compound against *A. baumannii* 17978 and MDR *A. baumannii* 5075. $EC_{50}$ and $EC_{90}$ were calculated for *A. baumannii*. Micromolar concentration $EC_{50}$ and $EC_{90}$ were lower of *A. baumannii* 17978 compared to *A. baumannii* 5075. From the list, it appears that $[Ru(5,5'\text{-dmb})_2(IP\text{-}1T)]Cl_2$ is the most effective molecule to treat the infamous disease *A. baumannii* since it has the lowest $EC_{50}$ and $EC_{90}$ values **(Figure 23)**. Additionally, as we can see, this chemical also had the greatest phototherapeutic indices. Interestingly, this compound only possesses one thiophene ring in its structure. To better understand we have introduced activity plot dose response curves of $EC_{50}$ values using logarithmic scale **(Figure 24)**. Three has been a shark contrast between the dark and visible curves. Although the potency of the compounds is not great against other ESKAPE pathogens.

**<u>Isolation of resistant mutants</u>**

We wanted to find out the mechanisms behind this activity and isolated some resistant mutant growing on LB agar plates in presence of two times MIC of compound 8. The fact that no mutation was found after whole genome sequencing, however, the negative result just serves to emphasize how difficult it is to evolve resistance to photosensitizer.

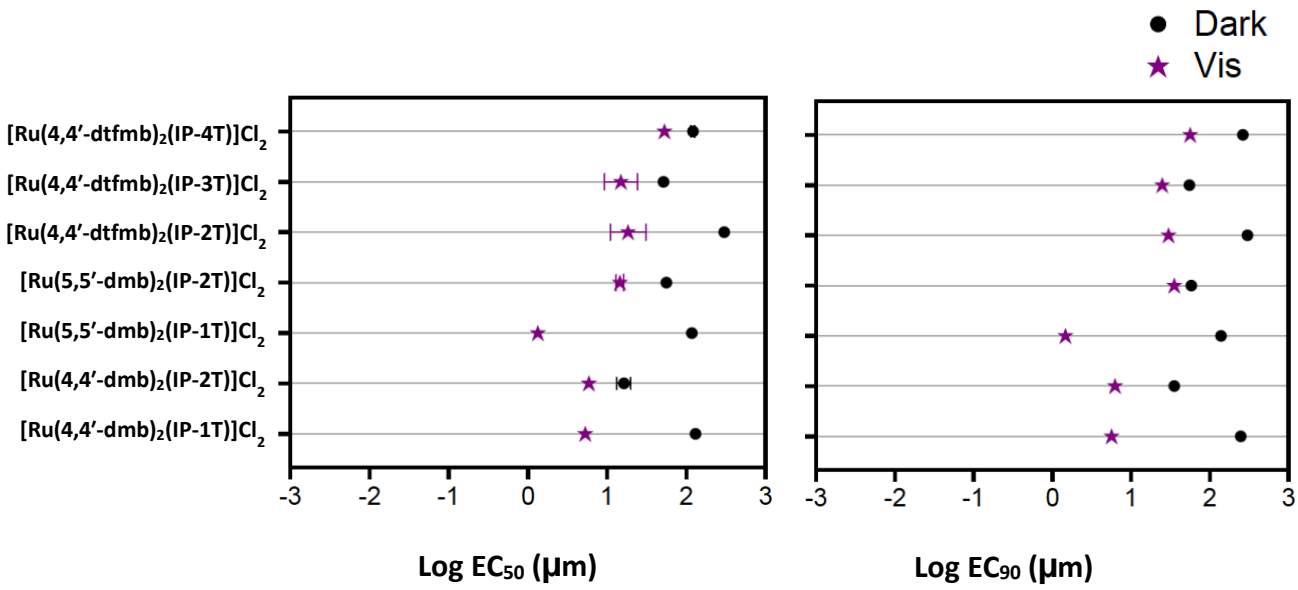



**Figure 23: Activity plots of Log $EC_{50}$ and Log $EC_{90}$ of selected seven compounds against *A. baumannii* 5075 both in Light and dark**

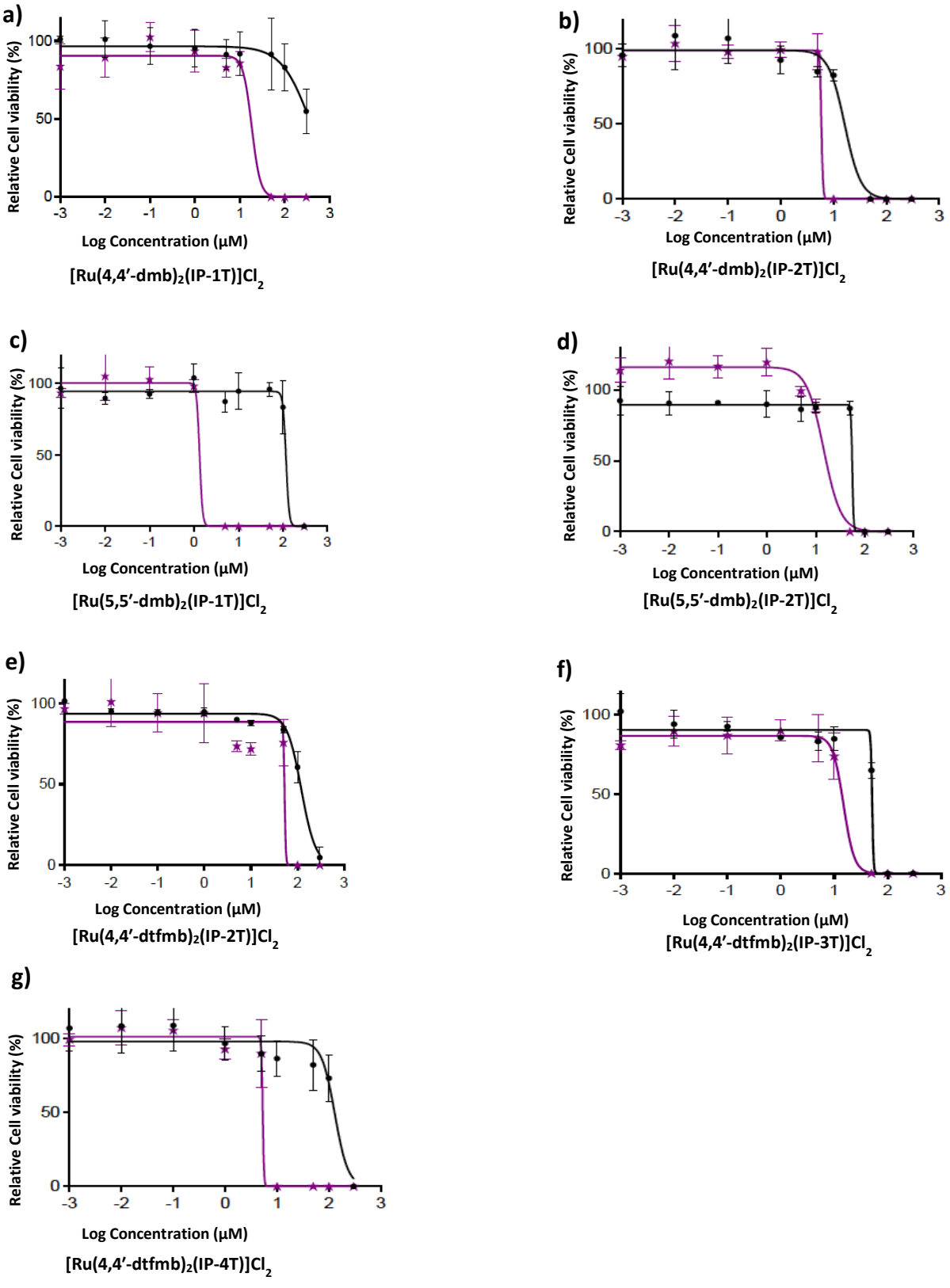



**Figure 24: Dose response curves for seven selected compounds against *A. baumannii* 5075.**
(Inoculum: $1x10^6$ CFU $ml^{-1}$; Irradiation: 100 $Jcm^{-2}$; Incubation: 20hrs)

**Discussion**

Antimicrobial PDT (aPDT) has potential to substitute for the widening gaps with our current antibiotic regimen[175–179]. In fact, the rising proliferation of multidrug-resistant (MDR) microorganisms may be overcome since aPDT does not require any contact with a specific molecular target and may be more resilient than the latter[176–178,180]. An alternative strategy is represented by light-triggered antimicrobials, which upon photoactivation might overwhelm the bacterial survival mechanism by releasing an instantaneous and non-targeted burst of reactive molecular species (RMS)[118,120]. Photoantimicrobials should not be mistaken with biocides because they are typically non-toxic compounds. They kill microbes more quickly and at much lower concentrations than biocides due to their photocatalytic mode of action. A single photosensitizer molecule can generate as many as 10,000 molecules of singlet oxygen before it is destroyed, and clinical management is easily accomplished by regulating the dose of light administered[118]. We made use of this photosensitizer's potential to treat a serious nosocomial infection, *A. baumannii* for which the development of antibiotics is crucial[3].

Complexes of ruthenium-based metal have demonstrated important biological and medical uses, such as anticancer and antibacterial agents. Multiple oxidation states, efficient light absorption, ease of absorption and excretion from the body, and a variety of binding types are only a few of their special qualities[181–186]. Studies on the Ru-dppz compounds' antibacterial properties showed that $[Ru(phen)_2(dppz)]^{2+}$and $[Ru(2,9\text{-}Me2phen)_2(dppz)]^{2+}$, show promising effectiveness against Gram-positive (+) microorganisms[187]. $[Ru(bb7)(dppz)]^{2+}$, a lipophilic compound, has bactericidal action against both Gram (+) and Gram (-) microorganisms[122]. The complexes mer-[RuIII(2-bimc)$_3$] H2O and cis-[$Ru^{IV}Cl_2(2,3\text{-}pydcH)_2$] 4H2O exhibit bacteriostatic activity against both Gram (+) and Gram (-) bacteria, according to research by Wawrzycka and colleagues[188]. FDA has not even given one ruthenium-based antimicrobial agent approval for clinical use, despite the promising antibacterial characteristics of Ru-based complexes. It has been demonstrated that two mechanisms—DNA damage and cytoplasmic membrane damage—are used by photodynamic antibacterial drugs to cause bacterial fatalities.

In this work, we used Ru-based photosensitizers to treat *A. baumannii* therapeutically. We obtained some variants of a Ru-based photosensitizer that is now being tested in Phase II

clinical trials for the treatment of non-muscle invasive bladder cancer (NMIBC), and we assessed their potential as antimicrobials. Seven candidate compounds were left after the compounds were screened by modifying their ligands. Under visible light, the compound [Ru(5,5'-dmb)$_2$(IP-1T)]$Cl_2$ exhibits the greatest PIs and lowest $EC_{90}$. The remaining substances are likewise active against *A. baumannii*. We tested the compounds' effectiveness against MDR *A. baumannii* 5075 strains, and it seems promising because they show antimicrobial activity. When we tested other ESKAPE pathogens with these compounds, efficacy was lower, suggesting they work better against *A. baumannii* strains. We were unable to determine the mechanisms behind the photosensitizers' action since the isolates that grew two times MIC of these drugs showed no signs of mutation. However, we hypothesize that these compounds may penetrate *A. baumannii* cell envelop more effectively than other ESKAPE pathogens. They may intercalate in DNA after penetrating and induce distortion[189].

The results of this study provide a novel solution to the problems we now have in treating *A. baumannii* infection. Since these substances are triggered by light, treating infections could benefit from this. To make sure they are safe to employ, we still need to assess these chemicals' cytotoxicity in various human cell lines. An additional area that will highlight the effectiveness of these drugs is *in vivo* research with *A. baumannii* infections.

# CHAPTER 5

## OM lipoproteins contribute to the rigidity of LOS-deficient cell envelope in *A. baumannii*

**Abstract**

The Gram-negative outer membrane (OM) serves as a barrier against several hazardous elements, including biocides, antimicrobial agents, and the host immune system. LOS is enriched in the outer leaflet of the OM, a highly conserved feature in Gram-negative bacteria. LOS is a glycolipid that shields the cell from harmful environmental compounds. Not only does LOS act as a barrier, but recent studies showed that it also contributes to mechanical stability in coordination with the peptidoglycan network to counter the turgor pressure. While LOS is important for Gram-negative survival, *A. baumannii* has the unique ability to mutationally inactivate LOS biosynthesis, which promotes resistance to several clinically important antibiotics. In this study, we explored how the cell envelope mechanical stability was maintained in the absence of LOS. This work characterizes two lipoproteins, Lpp1 and Lpp2, that showed overexpression in LOS-deficient isolates relative to wild type. We show evidence that the lipoproteins are covalently cross-linked with peptidoglycan via their c-terminal lysine residue. When the genes encoding lipoproteins were deleted, 50 LOS-deficient recovery on colistin was significantly reduced. We also found that Lpp1 and Lpp2 are expressed in a growth-dependent manner and play diverse roles in maintaining cell shape in various metabolic conditions. In *lpp1* and *lpp2* mutants, these *A. baumannii* cells shed considerably more OM vesicles, indicating a envelop defect. We show that elevated expression of Lpp1 and Lpp2 help to compensate for the loss of LOS and restore OM stiffness. Although it is still unclear what regulates the crosslinking of lipoproteins to PG.

**Introduction**

The Gram-negative cell envelope is made up of an outer membrane (OM), a peptidoglycan cell wall, and an inner (cytoplasmic) membrane[190]. While the peptidoglycan cell wall confers mechanical integrity to the envelope[191–193], recent studies have shown that the Gram-negative outer membrane coordinates with the peptidoglycan network to counter the internal turgor pressure equally, if not more than the cell wall .

*A. baumannii* pose particular concern in healthcare settings. Many strains can survive without LOS[19], which was thought to be essential for Gram-negative survival. This can result from genetic mutations that inactivate the lipid A biosynthetic pathway. LOS deficiency in *A. baumannii* has been associated with altered antibiotic susceptibility[20,46,194]. It is unknown what fortifies *A. baumannii*'s outer membrane in the absence of LOS. The production of phospholipids with shorter acyl chains that reduce the membrane's overall hydrophobicity is one reason[79],while increased expression of important poly-N-acetylglucosamine (PNAG) genes is another[79,195]. Furthermore, a prior study from our lab found that LOS-deficient *A. buamnanii* upregulated several putative OM lipoproteins and genes that encode components of the lipoprotein transport pathway [16,17,20]. It is likely that the OM lipoproteins contribute to the outer membrane's stiffness and serve to make up for the absence of LOS.

The Gram-negative OM also contains OM lipoproteins and OMbeta proteins (OMPs)[196]. Bacterial lipoproteins play a key role in Gram-negative cell envelope formation. Lipoproteins are involved in a variety of essential and non-essential processes, including OM-peptidoglycan linkage[197,198], peptidoglycan synthesis[199,200], pili and flagella assembly[200,201], and protein and polysaccharide secretion[202,203]. Although the term 'lipoprotein' is commonly used to denote a non-covalent association of proteins and lipids, bacterial lipoproteins are classified as lipid-anchored proteins[92]. Lipoproteins are highly expressed, with around 1 million copies per cell[204]. It was recently discovered that ÓE, an important sigma factor that causes a stress response when -barrel folding or LPS targeting to the OM fails, regulates lipoprotein production[205]. Under standard growth conditions, lipoproteins can act as an OM tether, to keep it from separating from the cell[206,207]. Braun and Bosch reported a C-terminal lysine residue linked to the peptidoglycan through its ε-amino group[208]. A lipoprotein variant missing the C-terminal lysine

share most of the phenotypes of the lipoprotein mutant, such as production of OM blebs and envelop defects[209,210]. These studies emphasize the importance of lipoproteins in providing the single covalent link between the OM and the peptidoglycan layer[209,210].

In *E. coli*, three LD transpeptidases (LD-TPases), LdtA, LdtB, and LdtC, catalyze crosslinking of lipoprotein to the peptidoglycan(PG)[211]. Lpp is highly expressed lipoprotein, with around 1 million copies per cell[204]. Lpp-PG crosslink production is almost abolished in cells lacking the LdtB enzyme[211].LdtJ and LdtK are two putative LD-TPases encoded by *A. baumannii*, were essential when LOS biosynthesis is inactivated. LdtJ from *A. baumannii* generates LD cross-links and D-amino acid insertion onto stem peptides whereas the second homolog, ElsL or LdtK, was not necessary for L,D-cross-linking[143,212]. Kang et al. hypothesized ElsL to cross-link OM lipoproteins to peptidoglycan[143]. ElsL still prefers tetrapeptide substrates, but it is the first known YkuD-containing enzyme that does not have a signal sequence and is active in the cytoplasm[133]. Despite the fact that ElsL shares structural similarities with LdtB in *E.coli*, it is unlikely to contribute to lipoprotein crosslinking due to its subcellular location[133].

The first comparative examination of transcriptional regulation in the absence of lipid A/LOS was made possible by a large-scale transcriptomics research conducted on several *A. baumannii* strains[20]. 38 unique gene clusters were either up- or down-regulated in the four strains compared to their LOS-producing parent. Putative lipoproteins and lipoprotein transport pathway genes are among the conserved pathways that have been shown to be downregulated in various popular lab strains of *A. baumannii*[20]. In this study, we characterized the lipoproteins upregulated in LOS deficient *A. baumannii* and explored their role in cell envelope stability. *A. baumannii* strains ATCC 19606 encodes *HMPREF0010_02744* & *HMPREF0010_01944* which we named as *lpp1* and *lpp2.* These genes were highly upregulated when LOS biosynthesis is inactivated. The lipoproteins appear to be crucial because knockout mutants are not isolated in LOS deficient strain selection. The covalent link between the cell wall and the OM, mediated by C-terminal lysine, is critical for stability. Overall, lipoproteins compensate for LOS and contributed to the maintenance of a stiff OM.

**Materials and Methods**

Bacterial strains and growth

*A. baumannii* ATCC 17978, ATCC 19606 and lipoprotein knockout strains were cultivated aerobically on Luria- Bertani (LB) agar at 37°C from frozen stocks. Unless otherwise specified, antibiotics were used at the following concentrations: Kanamycin (25 mg/L), meropenem (10 mg/L), and tetracycline (10 mg/L).

Construction of genetic mutants

*A. baumannii lpp1, lpp2,* and *lpp1lpp2* mutants were constructed using the recombination-mediated genetic engineering as described previously[142,143]. 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 *A. baumannii* strain ATCC 17978 expressing pREC$_{Ab}$ (pAT03). Transformants were recovered in Luria broth and plated onto LB agar supplemented with 7.5 mg/L kanamycin. All genetic mutants were confirmed by PCR. Following the isolation of genetic mutants, the pMMB67EH::REC$_{Ab}$ Tet$^r$ plasmid was removed as described previously[142]. Isolated mutants were grown on LB agar supplemented with 2 mM nickel (II) chloride (NiCl2) and replica plated onto LB agar supplemented with kanamycin or tetracycline. The loss of the pMMB67EH::REC$_{Ab}$, Tet$^r$ plasmid in mutants susceptible to tetracycline and resistant to kanamycin was confirmed using PCR. To excise the chromosomal insertion of the kanamycin resistance cassette, cured mutants were transformed with pMMB67EH carrying the FLP recombinase (pAT08) and plated onto LB agar supplemented with tetracycline and 2 mM isopropyl b-D-1-thiogalactopyranoside (IPTG) to induce the expression of FLP recombinase. The successful excision of the kanamycin resistance cassette was confirmed using PCR. The Lpp1 and Lpp2 complementation vectors were constructed by amplifying the *lpp1, lpp2* from *A. baumannii* ATCC 17978 gDNA and cloned into the KpnI and SalI restriction sites in the pMMB67EHknR plasmid. The resulting pLpp1 and pLpp2 plasmids were transformed into the respective mutants and induced with 2 mM IPTG for complementation.

Isolation of LOS deficient *A. baumannii* colony

Isolation of LOS deficient *A. baumannii* colonies was done as previously described[20] with slight alterations. Briefly, cultures were grown to mid-logarithmic growth phase or stationary phase with or without antibiotics. One milliliter of optical density at 600nm ($OD_{600}$) of 1.0 (~ $10^9$ CFU) was collected via centrifugation at 1,500 X g. Cells were washed with 1ml of Luria broth and plated on LB agar supplemented with 10mg/ml of colistin. Isolated colonies were picked and replicated on LB agar supplemented with vancomycin (5mg/ml) and LB agar supplemented with colistin (10mg/ml). Colonies sensitive to vancomycin but resistant to colistin were deemed as LOS deficient.

Peptidoglycan isolation

Biological replicates were grown to mid-logarithmic or stationary phase in 400 mL of Luria broth. Cells were centrifuged (Avanti JXN-26 Beckman Coulter centrifuge and Beckman Coulter JA-10 rotor) at 7,000 g for 0.5h at 4°C, resuspended in chilled 6 mL PBS, and lysed via dropwise addition to boiling 8% sodium dodecyl sulfate (SDS). PG was further purified as previously described[213]. Briefly, muropeptides were cleaved from PG by Cellosyl muramidase (Hoechst, Frankfurt am Main, Germany), reduced with sodium borohydride, and separated on a 250- by 4.6-mm 3-mm Prontosil 120-3-C18 AQ reversed-phase column (Bischoff, Leonberg, Germany). The eluted muropeptides were detected by the absorbance at 205 nm. Eluted peaks were designated based on known published chromatograms [20,143,214]; new peaks were analyzed by MS/MS, as previously described[20].

Immunoblot assays

Lpp1, Lpp2, and RpoA polyclonal antibodies were generated (Thermo Fisher Scientific) from rabbit immunization with peptide fragments. Briefly, a 20-amino-acid peptide from Lpp1, Lpp2, and RpoA predicted to be solvent exposed were selected from the primary sequence of *A. baumannii* ATCC 17978, synthesized, and used to generate each specific antibody from rabbits. The collected serum was tested for reactivity in an enzyme-linked immunosorbent assay (ELISA) with peptide fragments and via Western blotting against whole-cell lysates. All Western blot analyses were performed using 4 to 12% Bis-Tris 10-well protein gels (Invitrogen) and NuPage morpholineethanesulfonic acid (MES) SDS running buffer (Novex). Gels were transferred with

NuPage transfer buffer (Novex) to 0.45-mm polyvinylidene difluoride (PVDF) membranes (Amersham Hybond). All blots were blocked in 5% milk and 1x Tris-buffered saline (TBS) for 2 h. For primary rabbit antisera, anti-Lpp1 and anti-RpoA were used at a 1:50 and 1:750 dilution respectively, while anti-Lpp2 was used at 1:500. Anti-rabbit horseradish peroxidase (HRP) secondary antibody was used at 1:10,000 (Thermo Fisher Scientific). The penta-his primary anti-mouse antibody was used at 1:500 (Invitrogen). Anti-mouse HRP secondary antibody was used at 1:10,000 (Invitrogen). SuperSignal West Pico Plus (Thermo Fisher Scientific) was applied to detect relative protein concentrations.

β-Galactosidase assay

β-galactosidase activity was measured as described previously[215]. Briefly, cells harboring P*lpp1-lacZ* and P*lpp2-lacZ* were diluted 1:100 from overnight cultures in Luria broth (LB), then incubated at 37°C. 2 mL cells were harvested at $OD_{600}$ = 0.6 and the pellet were resuspended with permeabilization solution (60 mM $Na_2HPO_4{\cdot}2H_2O$, 40 mM $NaH_2PO_4{\cdot}H_2O$, 10 mM KCl, 1 mM $MgSO_4{\cdot}7H_2O$, 50 mM β-mercaptoethanol) for 30–45 min at room temperature. Then, 200 µl of substrate (4 mg/ml O-nitrophenyl-β-d-galactoside, 50 mM β-mercaptoethanol) were added. The mixture was further incubated at 28°C until the yellow color developed. Five hundred microliters of 1 M $Na_2CO_3$ were added to stop the reaction, and the optical density was measured at 420 nm and 550 nm for each tube. The standardized amount of β-galactosidase activity was reported in Miller units. The ratio of P*lpp1-lacZ* and P*lpp2-lacZ* induction was calculated relative to the basal level in a WT strain. Bar graphs with corresponding statistical analysis were prepared using Prism 9.

Isolation of outer membrane vesicles

Outer membrane vesicles were isolated as described previously[143]. Briefly, cultures grown overnight were backdiluted to an $OD_{600}$ of 0.01 in 100 mL Luria broth and grown to stationary phase at 37°C. Cultures were then pelleted at 5,000 g for 15 min at room temperature, and the supernatant was filtered through a 0.45-mm bottle-top filter. The filtered supernatant was ultracentrifuged (Sorvall WX 801 ultracentrifuge with an AH-629 swinging-bucket rotor) at 151,243 g for 1h at 4°C. Following a final ultracentrifugation step, the outer membrane vesicle pellet was resuspended in 500 mL cold membrane vesicle buffer (50 mM Tris, 5 mM NaCl, 1 mM

MgSO4 [pH 7.5]). The isolation of outer membrane vesicles was repeated three times in duplicate; one representative data set is reported.

Quantification of total outer membrane vesicle proteins

A Bradford assay was used to determine outer membrane vesicle protein concentrations, as previously described[143]. To generate a standard curve, bovine serum albumin (BSA) was diluted at 0 to 20 mg/mL in Pierce Coomassie Plus assay reagent (Thermo Fisher) to a final volume of 1 mL. Outer membrane vesicles were diluted at 2, 5, 10, 15, and 20 mL in reagent to a final volume of 1 mL. A microplate spectrophotometer (Fisherbrand AccuSkan) was used to measure the absorbance ($OD_{595}$) of the standard and samples in a 96-well plate (BrandTech). Protein concentrations were determined by comparing the optical densities of the samples to the standard curve plotted in Microsoft Excel, and final quantifications were graphed in GraphPad Prism 9. Experiments were reproduced three times from each outer membrane vesicle isolation, and one representative data set is reported.

Ethidium bromide permeability assay

Permeability assays were done as previously described[216],with slight modifications. Briefly, cultures were grown overnight in 5 mL BHI medium, normalized, and back diluted (1:10) in BHI medium with and without meropenem. Cultures were withdrawn at 0, 6, and 12 h; washed 3 times with PBS; and normalized based on the OD600. One hundred eighty milliliters of the cultures were added to a 96-well black plate, and 6 mM EtBr was added immediately before fluorescence measurements. The relative fluorescence units (RFU) were analyzed using a synergy multimode plate reader (530-nm excitation filter, 590-nm emission filter, and 570-nm dichroic mirror). The temperature was adjusted to 25°C, and the results were read at 15-s intervals for 0.5 h. Assays were repeated three times in triplicate; one representative data set is reported. The mean RFU for each sample were calculated and plotted using Prism 9. Experiments were reproduced three times, and one representative data set is reported.

## Results

### Lpp1 and LPP2 are attached to the peptidoglycan

We examined the peptidoglycan (PG) attached proteome of *A. baumannii*. We grew cells to stationary phase and boiled the cells and treated the the lysates with sodium dodecyls sulphate (SDS). The lysates were run in and HPLC column and read by liquid chromatography. we determined protein attached to peptidoglycan using mass spectrometry. We used bioinformatic analysis to identify each protein that was abundant in the samples to create a peptidoglycan-attached proteome in no stress conditions vs under OM tress. We compared the proteomes of stess vs no stress and observed more than 600 proteins are common between them (**Figure 25a**). Lpp1 was detected in both no stress and stress conditions, whereas the, Lpp2 was only attached to PG in stress. Interestingly, both lipoproteins have characteristic C-terminal lysine residue that could covalently link the OM to peptidlgycan[217] **(Figure 25b)**. We are curious to see whether these lipoprotein mutants can produce LOS deficient colony. Interestingly, *lpp1* mutant could not recover any LOS deficient colony. After complementation with pLpp1, the cell can partially restore the wild phenotype that suggested the essentiality of Lpp1 to produce this LOS deficient colony. However, when the C-terminal lysine mutant of pLpp1 is complemented no LOS deficient colonies were recovered and behaved like the *lpp1* mutant. This validates the claims that lipoprotein variants missing the C-terminal lysine behave like lipoprotein mutants[209,210]. Similarly, *lpp2* mutants showing similar phenotype where the crosslinking of *lpp2* with peptidoglycan is important for *A. baumannii* to produce LOS deficient colony. Therefore, the findings suggest here under stress LOS deficient *A. baumannii* remodel the cell envelope by physically tethering lipoproteins between OM and PG.

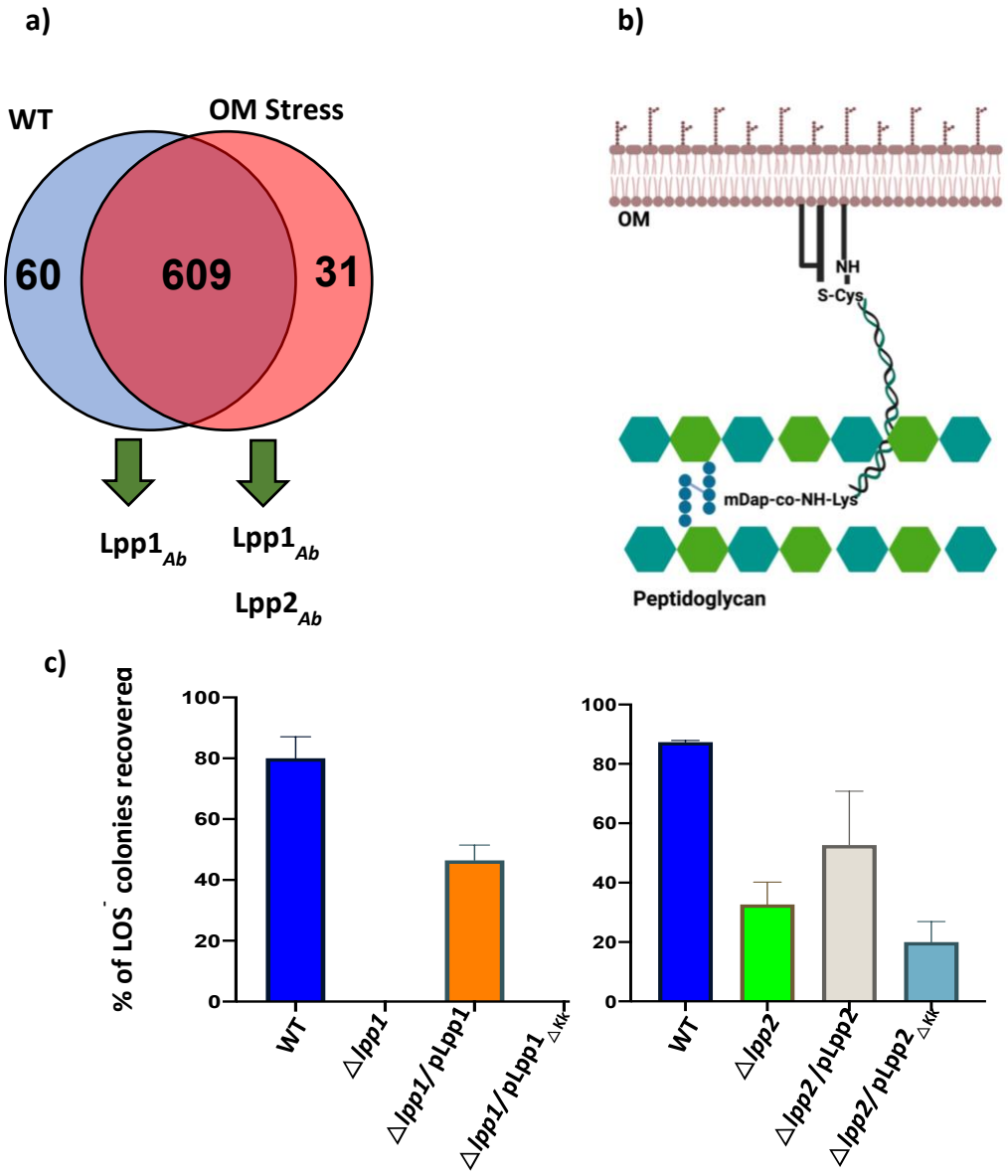



**Figure 25: PG attached proteome analysis. a) Comparison of PG attached proteome between WT vs OM stressed cells, b) Illustration of *lpp* crosslinked with PG using characteristics C terminal lysine residue, c) Bar diagram depicting % of isolated LOS deficient colony between WT vs *lpp* mutants and complementation.**

## Biochemical validation of lipoprotein attachment to peptidoglycan

Lipoprotein attachment in *E. coli* is dependent on C-terminal arginine-lysine (RK) residues[218]. With the exception of the C-terminal Lys-Arg dipeptide, the protease pronase E used in PG purification eliminates contaminating proteins and the majority of the covalently attached Lpp; the remnant dipeptide is employed in PG analysis to quantify the quantity of PG-attached Lpp in strains[213]. Same way, Braun and Rehn discovered that after treating isolated PG sacculi

with trypsin, a lysine residue remained covalently linked to approximately one out of every ten repeating units[219]. Therefore, we digested muropeptides of *A. baumannii* wild type and lipoprotein mutants with trypsin that leaves the tri-lysine residue and we have seen the presence Tri-lysine in wildtype. Muropeptide profiling using HPLC showed the absence of l-lysine peak in *lpp* mutants **(Figure 26)**. As a result, we concluded that Lpp1 and Lpp2 attach to PG in *A. baumannii,* and the attachment mediated by the tri-lysine residue.

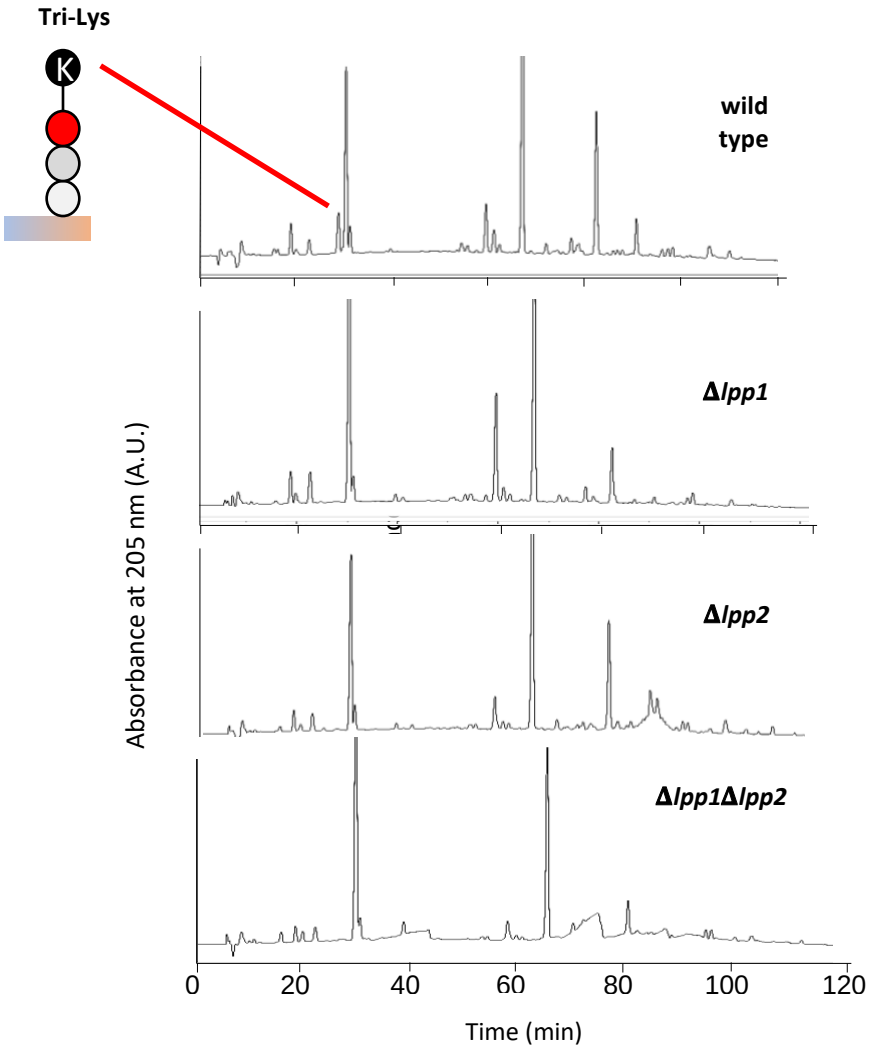



**Figure 26: Chromatogram of muropeptides analyzed using HPLC. The panels depicting the proteins present after trypsin digestion. Digested fragments are run in HPLC column and respective peaks are determined based on mass to charge ratio and only WT shows the presence tri-lysine peak whereas lipoprotein knockout stains didn't.**

**Lpp2 expressed in a growth-dependent manner**

A pathogen's growth phase is thought to alter the production of virulence factors[220]. The cell shrinks as it reaches stationary phase, and the DNA/protein ratio is stated to rise during the transition to stationary phase[221]. The distinctive proteins generated in the stationary phase are typically required for bacterial survival[222]. We are curious whether these lipoproteins expressed in a growth dependent manner. We isolated the whole cell lysate from different growth phase of wild type *A. baumannii* and run them on NuPage gel using Anti-Lpp1 and Anti-Lpp2 antisera. It appeared that Lpp2 expressed in the later phage of the growth whereas Lpp1 constitutively expressed regardless of the growth phase **(Figure 27b)**. To further validate our claim, we used β-Galactosidase assay, a colorimetric assay to assess the transcript level activation of *lpp1* and *lpp2*. The respective promoter of *lpp1* and *lpp2* was fused with LacZ and the strength of the promoter was quantified. The colorless ONPG substrate is converted into galactose and the chromophore o-nitrophenol by β-galactosidase, resulting in a vivid yellow solution. The amount of substrate transformed at 420 nm can be determined by measuring the solution's β-galactosidase activity with a spectrophotometer or a microplate reader. We have noticed that Lpp2 expressed highly in the stationary phage compared to the exponential phase **(Figure 27c)**.

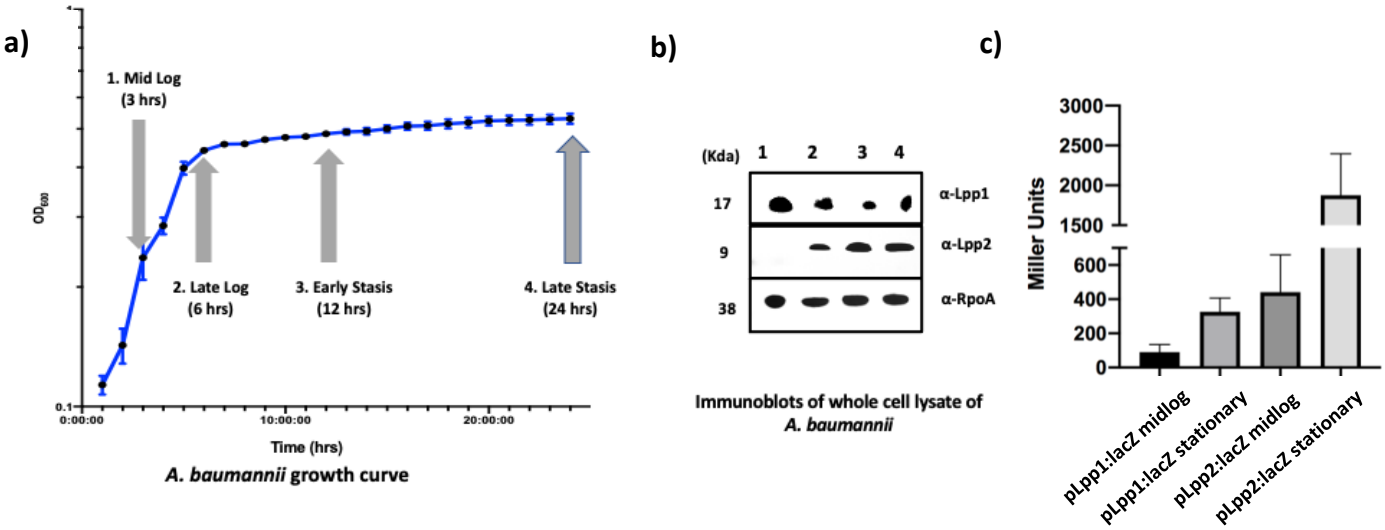



**Figure 27: Growth dependent activation of Lpp1 and Lpp2. a) *A. baumannii* ATCC 17978 growth curve and the hour of incubation when the whole cell lysates are collected, b) immunoblots illustrating the expression of Lpp1 and Lpp2 with loading control RpoA, c) Expression of promoters of *lpp1* and *lpp2* using β-Galactosidase assay.**

**Lipoproteins contributes to OM stability**

In *E. coli lpp* mutants develop OM blebs, suggesting that Lpp acts as a tether for the OM under normal growth circumstances, preventing it from tearing away from the cell. Outer membrane vesicles (OMV) are biologically relevant buds derived from the cell envelope of Gram-negative bacteria. In most Gram-negative bacteria the OM is covalently linked to the peptidoglycan cell wall, So, production of OMVs therefore must rely on dissociation of the OM from the periplasmic peptidoglycan layer which is an indication of unstable outer membrane[223]. We used Bradford assay to determine the total protein concentration of these isolated outer membrane vesicles. The Bradford assay, a colorimetric protein assay, is based on an absorbance shift of the dye Coomassie brilliant blue G-250. Measurement of the protein concentration using Bradford assay is based on a shift in absorption spectrum of the Coomassie Brilliant Blue G-250 dye. We have seen rapid shedding OMV in *lpp1* mutant compared to WT which further supports of our idea of importance of *lpp* in providing stability of outer membrane **(Figure 28a)**. We also saw hypervesiculation of *lpp2* as well. Additionally, we investigated the permeability of the OM using ethidium bromide (EtBr). It appears that the lipoprotein mutants are more permeable than the WT underscore their potential **(Figure 28b)**. So, the lipoproteins are important for stability of the *A. baumannii* cell envelope.

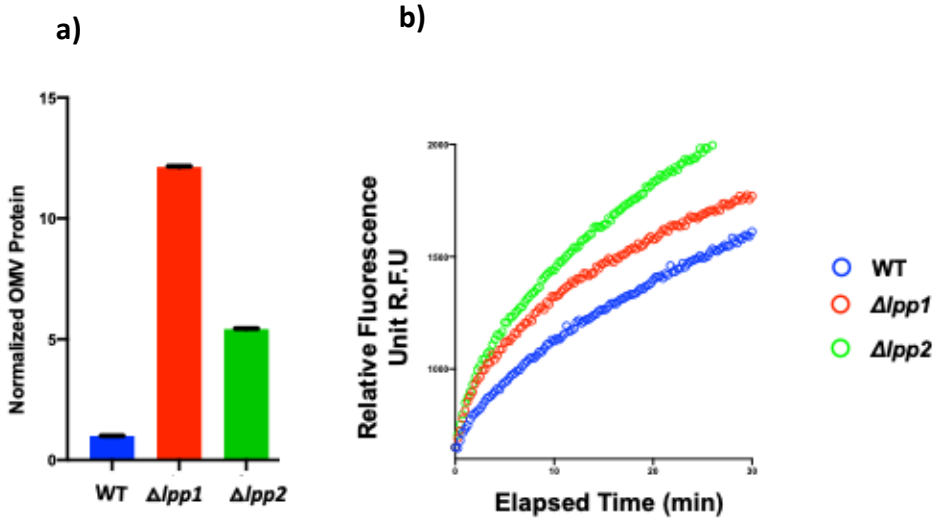



**Figure 28: Evaluating the OM inigrity. a) Quantification of total protein in isolated OMVs using bradford assay, b) Ethidium bromide assay showing permabliity of OM in *lpp* mutants**

**Discussion**

It's not always the case that members of a particular genus or even species can survive in the absence of lipid A and LOS. Only over 50% of *A. baumannii* isolates evaluated for LOS deficiency were able to survive without LOS, which underlines the vast dearth of understanding regarding signals and physiological requirements for inactivating lipid A biosynthesis[20]. Despite the recent improvements, there are still significant information gaps regarding LOS-deficient membranes, their control, and the processes of membrane fortification in *A. baumannii*. In the absence of LOS, we have for the first time described an alternate membrane structure of the OM of *A. baumannii* by packing the space with surface-exposed lipoprotein. We used an -omics method to reveal the differences between the WT and LOS-deficient OM and found that the LOS-deficient OM had higher levels of lipoprotein expression[20].

Some outer membrane lipoproteins are covalently connected to the underlying PG network, which strengthens the *Escherichia coli* cell envelope by providing structural stiffness[224,225]. The first lipoprotein found to be covalently attached to peptidoglycan was Lpp[219]. However, nothing is known about the protein's functional significance despite its extreme abundance. *Salmonella, Pseudomonas*, and other gamma proteobacteria were shown to contain homologous PG-bound lipoproteins shortly after Lpp was discovered in *E. coli*[226]. Subsequent to LOS deficiency, ATCC strain 19606, a common type of strain, had anywhere from 5 to 25 elevated lipoproteins[20,195]. *E. coli* cells missing Lpp have some phenotypes, including enhanced susceptibility to poisonous substances, chelating agents, and hydrophobic antibiotics such SDS, EDTA, deoxycholate, dibucain, and vancomycin[227–230]. Another critical function of Lpp in regulating the bacterial cell envelope's construction, particularly the IM-to-OM distance[210].

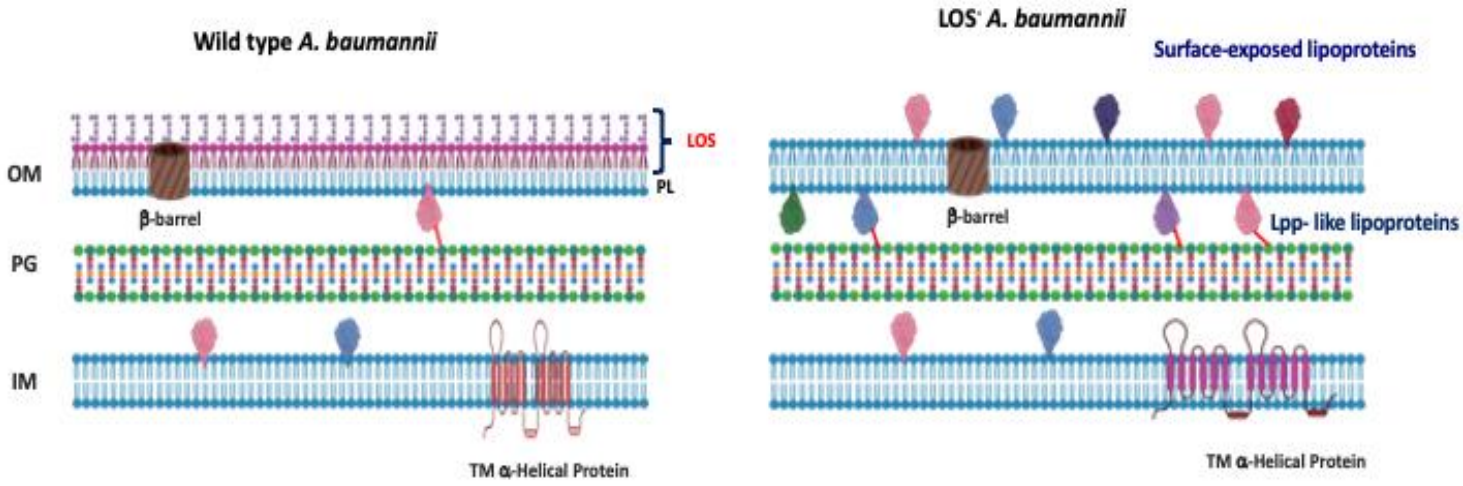



**Figure 29: Working model of LOS deficient OM. Surface-exposed lipoproteins make up for LOS located in the outer leaflet of OM.**

Following transcriptomic analysis, we have validated the role of Lpp proteins empirically. Lpp proteins are crucial in the structural orientation of LOS deficient outer membranes, as evidenced by *A. baumannii*'s inability to form any LOS deficient colonies in the absence of Lpp proteins. In the absence of Lpp proteins, *A. baumannii* considerably sheds more OM vesicles and is more permeable to EtBr, which may account for their reduced integrity. Our working model **(Figure 29)** illustrate the structural alteration where Lpp proteins are transported to OM to provide rigidity of the membrane. However, we provided enough evidence to hypothesize that Lpp proteins function to compensate for LOS absence, even if we were unable to identify what controls this signaling cascade. Although Lpp has been researched over five decades, it still has a few closely guarded mysteries. Such questions as where and when the crosslinking of Lpp to the PG occurs, whether the distribution of bound Lpp is homogenous throughout the cell axis and at the pole, and how cells regulate the ratio between the bound and free form of this protein are still being thoroughly examined. Like this, it would be fascinating to investigate what causes Lpp1 and Lpp2, two of the described lipoproteins, to activate in a growth-dependent manner when the PG is stressed.

# CHAPTER 6

# CONCLUSIONS and RECOMMENDATIONS

The investigations presented here are aimed at clarifying the processes that control *A. baumannii*'s stress response. What causes *A. baumannii* to resist environmental challenges brought on by dangerous chemicals has been a mystery for a very long time. Here, we describe a TCS called BaeSR and reveal that it can control one of *A. baumannii*'s recently discovered stress response pathways. We also proposed an alternate method of clinical management of *A. Baumannii* since there are paucity of effective antimicrobial drugs available,. Finally, We discussed how specifically *A. baumannii* reoriented its OM is when LOS biosynthesis is turned off.

Studies in chapter 3 described a unique signaling pathway mediated by BaeSR TCS. BaeSR is critical in *E.coli* as this TCS gets activated by cell envelope stress[87]. BaeSR expressed profoundly when LOS biosynthesis is turned off in *A. baumannii*, but their function is unknown[20]. The fact that *A. baumannii* did not produce any LOS-deficient colonies after baeSR was genetically deleted implies that the bacterium controlled certain vital survival processes. I utlizied RNA seq analysis to determine the regulatory pathways of BaeR. I overexpressed the BaeR gene, which is regulated by an induced promoter, and compared the results to an uninduced control in order to conclusively quantify the degree of transcriptome expression. With the induction of BaeR, all fourteen of the genes in the *paa* operon that are in charge of phenyl alanine catabolism are upregulated. It wasn't until recently that it was discovered that the *paa* operon is one of the ubiquitous pathways that is triggered by a variety of environmental stresses. The capacity of *A. baumannii* to respond to stress was hindered by disrupting PAA catabolism, which resulted in reduced antibiotic tolerance and hydrogen peroxide resistance. The study of this stress response may result in the creation of innovative treatments. In reality, a class of nonsteroidal anti-inflammatory medications known as PAA derivatives has FDA approval and may be used to treat multidrug-resistant *Acinetobacter* infections as antivirulence therapy. And ever since we now know that *paa* operon is regulated by BaeR this could potentially be used a target for therapeutic developement.

The notion that PAA is a crucial component in several signaling cascades is supported by the involvement of various regulators in the control of the *paa* operon. *A. baumannii* uses PAA in a signaling pathway. How PAA causes these physiological changes is the primary unanswered question. It is still unclear, though, how much cellular PAA would be required to mount neutrophil activation. While looking for a biochemical assay to evaluate the amount of cellular PAA generated in *baeR* knockout strains to the wild type level, I was unsuccessful. Additionally, I'm not sure how *paa* operon expression improves the survival of the *A. baumannii* colonies that lack LOS. The ability of the baeR mutant strains to survive in an infection model will be interesting since it may reveal whether or not the activation of the *paa* operon is related to the pathogencity of *A. baumannii*.

In chapter 4 i reported an alternative way to treat drug resistant pathogen *A. baumannii*. Antimicrobial resistance is a big issue, and research to combat it is solely focused on the development of new antibiotics. Unfortunately, this is not a long-term solution since microorganisms ultimately evolve resistance tactics. The fundamental reason for this is that antibiotics are directed at a necessary component or pathway of the bacterium. However, finding an agent with a multi-target strategy to killing the bacteria may be a long-term answer. Light activated compounds, often known as photoantimicrobials, are hardwired with mechanisms capable of challenging a variety of molecular microbial targets such as protein, lipids, and nucleic acid. Despite the fact that it has been available for a long time and is utilized in anticancer therapy, the phoodynamic inactivation of microorganisms remains underappreciated. I have screened a handful of this metal based photosensitizers against *A. baumannii* which is very hard to treat.

These photosensitizers showing excellend efficacy not against the lab strain but also against multi-drug resistant *A. baumannii* 5075. I cacluated their $EC_{50}$ and $EC_{90}$ values and phototherapeutic indices (PI) and observed that they are readily comparable with broad spectrum beta-lactum antibiotic meropenem. This clearly turned out to be a new and much more efficient way of treating *A. baumanni* where it is highly unlikely to develop resistance. To prove this point i even grow *A. baumannii* in presence of two times of minimum inhibitory concertation and patched them for few days and sequenced their whole genome. I did not encounter any

mutation in their genome. I see a lot of promise of use this as an alternative therapy against other notorious Gram-negative bacteria as well. Since its get activated by the visible light i propose an wound infection model will help us to determine efficacy of this compounds in vivo. This compound might also be very effective against dental pathogen which likely to get light exposure. Before concluding the efficacy it might be worthwile to see the toxicity agaist human epithelial cell line.

I discussed how A. baumannii maintains the integrity of its OM in the last data chapter, chapter 5, which is a hallmark for any Gram-negative bacterium. Previous research had shown that LOS deficient OM had higher lipoprotein expression than the wild type. However, the activities of the OM lipoproteins in *A. baumannii* are unclear, and I am working to find out. These lipoproteins were produced in the cytoplasm, developed in the cytoplasmic membrane, then transported to the OM through the lol route. I have proof that the crosslinking of peptidoglycan with OM keeps the membrane tight. When the bacterium is stressed, these lipoproteins known as Lpp1 and Lpp2 connect to PG. Without the lipoproteins the bacterial membrane stiffness is compromised and more permeable.

However, this is still unknown how what regulates the crosslinking of these lipoproteins. *E. coli* encodes LD trasnpeptidase that mediates this crosslinking. In *A. baumannii* there is no periplasmic LD transpeptidases characterized so far that could potentially do the crosslinking. LOS located in outer membrane is critical in mounting immune responses against *A. baumannii* and since these lipoproteins compensate for LOS might play the same role. We need to investigate wether the expression of this lipoproteins are a common phenomenon in case of stressful environment. Therfore, evalute the expression of lipoproteins expression in presence of OM targetting agents and cell wall targetting agents will enhance our understadning about their expression. Beta-galactosidase assay could be employed as the promoter of the respective lipoproteins fused with LacZ and the expression is quantified. I will conclude by saying that if the lipoproteins expression is readily increased by toxic substance then this will have a promise to be an ideal drug target. Infection model study with the lipoprotein mutants will picture whether this absence of lipoproteins have any deletorious effect on mounting immunre response.

Taken together, our findings reveal an intriguing phenomena about how the noxious gram negative bacterium *A. baumannii* exerts its signaling cascade under stress. I also explain how the LOS-deficient OM reorients itself to fulfill its required role. Finally, an alternate way to treating *A. baumannii* was proposed, which will be hopeful in the future and a fantastic response to the worry of antibiotic resistance.

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# CHAPTER 7

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