Investigation of the effects of polymymyxins on sub-proteome and lipidome of *K. pneumoniae*

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Drug Delivery, Disposition and Dynamics

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This thesis is dedicated

to my dearest parents, brothers and sisters,

to my beloved wife, Dalal;

and to my sons (Mohammed and Haider)

Contents

Abstract

Global health is clearly facing a serious problem arising from multi-drug resistant (MDR) Gram-negative bacteria. Cystic fibrosis (CF), which is lethal genetic disease, usually associated with bacterial infection caused by both MDR Gram-negative and positive bacteria, especially *Pseudomonas aeruginosa*. Patients with CF found to be at high risk because they repose on antibiotics regimen for a long period which results in the development of resistance to most of the clinically available antibiotics. Polymyxins, old antibiotics, considered as a last resort therapy of such infections. However, resistance to polymyxins can rapidly emerge with monotherapy. In addition, polymyxins are associated with high incidence of nephrotoxicity and neurotoxicity. Combination therapy of polymyxins with other antibiotics has the potential to minimise the emergence of resistance and ameliorate toxicity. In overview, this thesis aims to assess the novel synergistic combination of polymyxin B and silver nanoparticles (silver NPs) against MDR Gram-negative bacteria associated with CF and shed light on mechanistic effects of polymyxin on the outer membrane sub-proteome and lipidomes of *Klebsiella pneumoniae*.

In the first experimental chapter, the project aims to identify a superior *in vitro* polymyxin B combination with silver NPs against CF lung infection causative MDR Gram-negative bacteria. The combination was examined against polymyxin-susceptible and polymyxin-resistant Gram-negative CF isolates including *P. aeruginosa, Haemophilus influenzae, Burkholderia cepacia, Burkholderia pseudomallei, Stenotrophomonas maltophilia, K. pneumoniae and Acinetobacter baumannii*. Silver NPs displayed a range of activities against the tested isolates. In static time-kill studies, polymyxin B alone, silver NPs alone and the combination of polymyxin B and silver NPs were performed against two polymyxin-resistant *P. aeruginosa* CF isolates, one polymyxin-resistant *A. baumannii* isolate and one polymyxin-resistant *K. pneumoniae* isolate. polymyxin B monotherapy was ineffective against all isolates, while silver NPs showed some killing activity against *P. aeruginosa*. On the other hand, the combination demonstrated a significant synergistic killing activity against all four tested isolates. An artificial sputum media antibacterial assay was also employed to investigate the antibacterial activity of the combination

in CF relevant conditions. Under planktonic growth conditions, the combination and silver NPs monotherapy were effective against the polymyxin-resistant *P. aeruginosa* CF isolate FADDI-PA066. Whereas, under biofilm growth conditions, neither the combination nor the monotherapies were active.

Microorganisms, especially Gram-negative bacteria constitutively secrete outer membrane vesicles (OMVs) into the extracellular space. These vesicles play a role in bacterial virulence, inflammation, host immune stimulation, and antimicrobial resistance. In the second experimental chapter, comparative studies were performed on the proteome of OMVs isolated from both polymyxin-susceptible and -resistant *K. pneumoniae* isolates with and without polymyxin B (2mg/L) treatment. Polymyxin B treatment caused significant changes in the proteome of released OMVs, also the resistant strain produced OMVs proteins quantitatively differ from that of susceptible strain. Metabolic pathway analysis of the OMV proteome revealed that polymyxin B treatment of both susceptible and resistant *K. pneumoniae* isolates significantly reduced the expression of proteins (mainly enzymes) that participate in cationic antimicrobial peptide (CAMP) resistance pathways, β-lactam antibiotic resistance and quorum-sensing (QS) activities including iron uptake and detoxification.

In the third tier of the project, comparative lipidomics studies were performed on OMVs isolated from polymyxin-susceptible and -resistant *K. pneumoniae* isolates pre-treated with a clinically relevant concentration of polymyxin B (2mg/L). Transmission electron microscopy (TEM) and dynamic light scattering (DLS) were initially used to study the effect of polymyxin B on the particle size of OMVs derived from polymyxin B pre-treated polymyxin-susceptible and -resistant *K. pneumoniae* ATCC700721. TEM and DLS findings showed that OMVs secreted from polymyxin B treated strains were larger than those derived from control untreated strains. Lipidome analysis revealed that polymyxin B treatment resulted in production of OMVs rich in glycerophospholipis, fatty acyls and sphingolipids. Compare to untreated control, polymyxin B treatment of the polymyxin B susceptible strains significantly reduced most of these lipids in OMVs.

In the fourth tier of the project, comparative studies were performed on the outer membrane (OM) proteome of paired colistin-susceptible and extremely colistin-resistant *K. pneumoniae* ATCC 13883 strains to identify the key difference between outer membrane proteins (OMPs) of colistin-susceptible and –resistant strains. Notably, the OMPs (e.g. OmpA and W, non-specific DNA-binding protein and Iron-binding ferritin-like antioxidant protein) of resistant strain quantitatively differ from that of susceptible strain. On the other hand, OMPs profiling of both colistin-susceptible and –resistant *K. pneumoniae* ATCC 13883 strains, using SDS-PAGE gels, showed no difference in OM porin proteins, particularly the efflux pumps OmpK35 and OmpK36.

Overall, this thesis sheds new light on novel polymyxin-silver NP combinations for the treatment of CF associated chronic Gram-negative infections. Our lipidomic and proteomic data give a clear insight about the selective packaging of lipids and proteins in OMVs and OM of Gram-negative bacteria in response to polymyxin B treatment.

Declaration of Authorship

Declaration for thesis based or partially based on conjointly published or unpublished work

General Declaration

In accordance with Monash University Doctorate Regulation 17.2 Doctor of Philosophy and Research Master's regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes two original papers published in a peer-reviewed journal (**Chapter 2 and 4**). **Chapter 5** has been submitted as original manuscripts to peer-reviewed journals for publication. **Chapter 3** has not been submitted yet.The core theme of the thesis is to understand the synergistic activity of polymyxin combinations against MDR Gram-negative pathogens, and to investigate the mechanistic effects of polymyxin on sub-proteome and lipidomes of extremely-drug resistant (XDR) *Klebsiella pneumoniae*. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Department of Microbiology under the supervision of Professor Jian Li and within the Faculty of Pharmacy and Pharmaceutical Sciences, under the supervision of A/Prof. Tony Velkov.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research. In the case of **Chapters 2**, **3, 4** and **5**, my contribution to the work involved the following:

Thesis Chapter	Publication Title	Status *(published, in press, accepted or returned for revision, submitted)*	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution*	Co-author(s), Monash student Y/N*
2	A fresh shine on cystic fibrosis inhalation therapy: antimicrobial synergy of polymyxin B in combination with silver nanoparticles	Published	85%. Experimental design, experimental execution, data analysis, data interpretation, data visualisation and original draft preparation	1) Elena K. Schneider, Mathematical modelling and editorial review 1% 2) Meiling Han, Experimental design and editorial review 1% 3) Mohammad A. K. Azad, Supervision and editorial review1% 4) Maytham Hussein, Editorial review 1% 5) Cameron Nowell, Editorial review 1% 6) Mark A. Baker, Editorial review 1% 7) Jiping Wang, Editorial review 1% 8) Jian Li, Supervision, experimental design, data interpretation and editorial review 4% 9) Tony Velkov, Supervision, experimental design, data interpretation and editorial review 4%	Yes Yes Yes Yes Yes No Yes Yes Yes
4	Lipidomic composition of outer membrane vesicles from paired polymyxin-susceptible and -resistant *Klebsiella pneumoniae* clinical isolates	Published	85%. Experimental design, experimental execution, data analysis, data interpretation, data visualisation and original draft preparation	1) Xiaohan Hu, Editorial review 1% 2) Yan Zhu, Mathematical modelling and editorial review 1% 3) Meiling Han, Experimental design and editorial review 1% 4) Yu-Wei Lin, Editorial review 1% 5) Maytham Hussein, Editorial review 1% 6) Charlie Yao Da Dong, Editorial review 1% 7) Jian Li, Supervision, experimental design, data interpretation and editorial review 4% 8) Tony Velkov, Supervision, experimental design, data interpretation and editorial review	No Yes Yes Yes Yes Yes Yes

				5%	
5	A comparative study of outer membrane proteome between a paired colistin-susceptible and extremely colistin-resistant *Klebsiella pneumoniae* strains	Published	80%. Experimental design, experimental execution, data analysis, data interpretation, data visualisation and original draft preparation	1) Mark A. Baker, Editorial review 1% 2) Yan Zhu, Mathematical modelling and editorial review 1% 3) Meiling Han, Experimental design, experimental execution and editorial review 5% 4) Elena K. Schneider, Mathematical modelling and editorial review 1% 5) Maytham Hussein, Editorial review 1% 6) Daniel Hoyer, Editorial review 1% 7) Jian Li, Supervision, experimental design, data interpretation and editorial review 5% 8) Tony Velkov, Supervision, experimental design, data interpretation and editorial review 5%	No Yes Yes Yes Yes No Yes Yes

For **Chapter 2, 3, 4 and 5,** I have renumbered sections as well as reformatted references, abbreviations, nomenclature and writing conventions in order to generate a consistent presentation within the thesis.

Candidate signature: **Date: 29/08/2018**

[redacted]

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature: **Date:** 29/08/2018

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Publications

- Raad Jasim, Elena K. Schneider, Meiling Han, Mohammad A. K. Azad, Maytham Hussein, Cameron Nowell, Mark A. Baker, Jiping Wang, Jian Li, and Tony Velkov, A fresh shine on cystic fibrosis inhalation therapy: antimicrobial synergy of polymyxin B in combination with silver nanoparticles. *Journal of Biomedical Nanotechnology*. Volume 13, Number 4, April 2017, pp. 447-457(11).
- Raad Jasim, Mei-Ling Han, Yan Zhu, Xiaohan Hu, Maytham H. Hussein, Yu-Wei Lin, Qi (Tony) Zhou, Charlie Yao Da Dong, Jian Li and Tony Velkov, Lipidomic Analysis of the Outer Membrane Vesicles from Paired Polymyxin-Susceptible and -Resistant *Klebsiella pneumoniae* Clinical Isolates. Int J Mol Sci. 2018 Aug 10;19(8). pii: E2356.
- Raad Jasim, Mark A. Baker, Yan Zhu, Meiling Han, Elena K. Schneider, Maytham Hussein, Daniel Hoyer, Jian Li, Tony Velkov, 2018, A comparative study of outer membrane proteome between a paired colistin-susceptible and extremely colistin-resistant *Klebsiella pneumoniae* strains. *ACS Infect. Dis.*, 2018, *4* (12), pp 1692–1704.

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Glossary of Abbreviations

AMR	Antimicrobial resistance
ASM	Artificial sputum media
AZM	Azithromycin
CDC	Centres for Disease Control and Prevention
CPS	Capsule polysaccharide
CRE	Carbapenem-resistant *Enterobacteriaceae*
CRKP	Carbapenem-resistant *K. pneumoniae*
CBA	Colistin base activity
CMS	Colistimethate sodium/Colistin methanesulfonate
CF	Cystic fibrosis
Dab	Diaminobutyric acid
DLS	Dynamic light scattering
ECDC	European Centre for Disease Prevention and Control
ESBL	Extended-spectrum β-lactamase
FDA	Food and Drug Administration
GC	Gas chromatography
HPLC	High-performance liquid chromatography
KPC	*Klebsiella pneumoniae* carbapenemase
MDR	Multi-drug resistant
MIC	Minimal inhibitory concentration
MS	Mass spectrometry
NDM	New Delhi metallo-β-lactamase
NIH	National Institutes of Health
OM	Outer membrane
OMPs	Outer membrane protein
OMVs	Outer membrane vesicles
QS	Quorum-sensing
Silver NPs	Silver nanoparticles
TEM	Transmission electron microscopy
CFTR	Trans-membrane conductance regulator
XDR	Extremely-drug resistant

Chapter 1: General Introduction

1.1 Statement of the problem

The emergence of MDR Gram-negative bacteria has evolved into a major global health crisis. Lack of novel antimicrobial agents and the continuous dwindling antibiotic pipeline augment this problem. In spite the fact that resistance to Gram-positive MDR bacteria is considered as a part of this public issue, new effective antibiotics for fighting these organisms have been recently approved by the U.S Food and Drug Administration (FDA). The treatment of Gram-negative bacilli infections remains an outstanding obstacle. *P. aeruginosa, B. cepacia, B. pseudomallei, A. baumannii and K. pneumoniae* are among those highly resistant Gram-negative superbugs that cause serious infection especially with chronic diseases such as CF to which novel, effective antibiotics are urgently required. As a result, there has been an increase in the usage of old antibiotics, such as the polymyxins. Although, polymyxins are considered as a last line antibiotics for these organisms, the toxicity and emerge resistance could limit their use. Therefore, the combination of polymyxins with other antibiotics is an option to increase the efficacy and minimize the resistance to these antibiotics.

The optimal clinical application of polymyxins requires better understanding of their mechanism of action and bacterial resistance mechanisms. Omics studies (including proteomics, lipidomics and transcriptomics) are among the most efficient approaches for better understanding the mechanism of drug action and mechanism of bacterial resistance. These approaches are highly applicable nowadays to clarify the mechanisms of antibiotics-pathogen interaction. The central focus of this PhD thesis was to investigate the mechanism of the synergistic antibacterial activity of polymyxin B in combination with silver NPs against MDR pathogens that commonly associated with CF; and the mechanistic effects of polymyxin B on sub-proteome and lipidome of the extremely-drug resistant (XDR) *K. pneumoniae*.

1.2 Bad bugs, no drugs

Over the past few decades, antimicrobial resistance (AMR) has grown severely and the infections evoked by MDR bacteria are nowadays becoming an alarming public health crises as highlighted by many organisations such as the European Centre for Disease Prevention and Control (ECDC) and the US Centres for Disease Control and Prevention (CDC) (1-3). In addition to the shortage in antibiotic development and discovery **(figure 1.1)**, many other factors, such as inappropriate and overuse of antibiotics, in the clinic and in agricultural, animal and environmental products, have strongly contributed in the development and spread of AMR **(Figure 1.2)** (4-6). Disconcertingly, it has been predicted that the AMR will continue to increase and by 2050 would result in a massive increase in mortality rate (10 million people dying every year) and reduction in the global Gross Domestic Product (GDP) of about 2% to 3.5% **(Figure 1.3)** (7, 8). Recently, AMR was proclaimed as one of the major human health concerns (9). In addition, this antibiotic resistance is mainly resulted from the six highly resistant microorganisms simply known as "ESKAPE" (i.e. *Enterococcus faecium, Staphylococcus aureus, K. pneumoniae, A. baumannii, P. aeruginosa*, and Enterobacter species). These pathogens are associated with high mortality and morbidity rate due to their resistance to most available antimicrobial agents (10, 11). Moreover, The CDC in association with the FDA and the National Institutes of Health (NIH) have prioritized the MDR Gram-negative pathogens into three major categories "Urgent", "Serious" or "Concerning" threats (12). In early 2017, the world health organization (WHO) developed a priority list for antibiotic resistant pathogen to help in development and discovery of new and effective antibiotics, the pathogens were ranked into three priority criteria, critical (priority 1), high (priority 2) and medium (priority 3) (13).

Urgent and coordinated action of different stakeholders (i.e. policy makers, public health authorities, regulatory agencies, pharmaceutical companies and the research community) is required to tackle AMR (3). "10 × '20" initiative, a campaign has been lunched by the Infectious Diseases Society of America (IDSA) to develop and approve 10 effectively and safely administered antimicrobial agents by the year 2020 (14). This goal can be achieved by proper

worldwide collaboration of research community to improve the antibiotic development pipeline. Concomitantly, optimising the currently in use antibiotics would highly contribute in the reduction of antibiotic resistance (5). Therefore, research nowadays focus on the last-line antimicrobial drugs (polymyxins) to improve their efficacy and reduce their toxicity has taken priority (15). The main MDR pathogens targeted in this thesis are *P. aeruginosa, A. baumannii and K. pneumoniae*; therefore, the following sections will include an overview of these dangerous human pathogens.

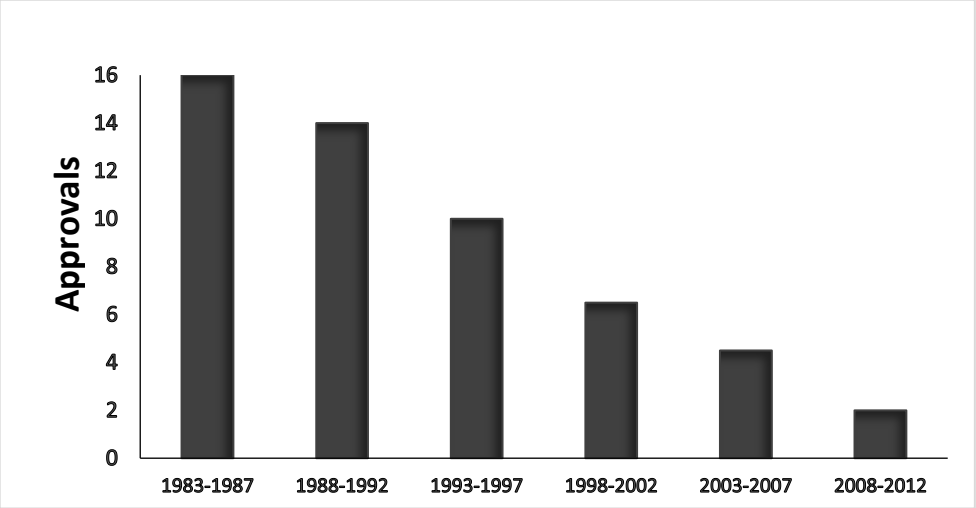

Figure 1.1 Number of new systemic antibiotics approved by US Food and Drug Administration every 5 years, from 1983 to 2012. (Figure modified from Spellberg *et al*. (7) with permission).

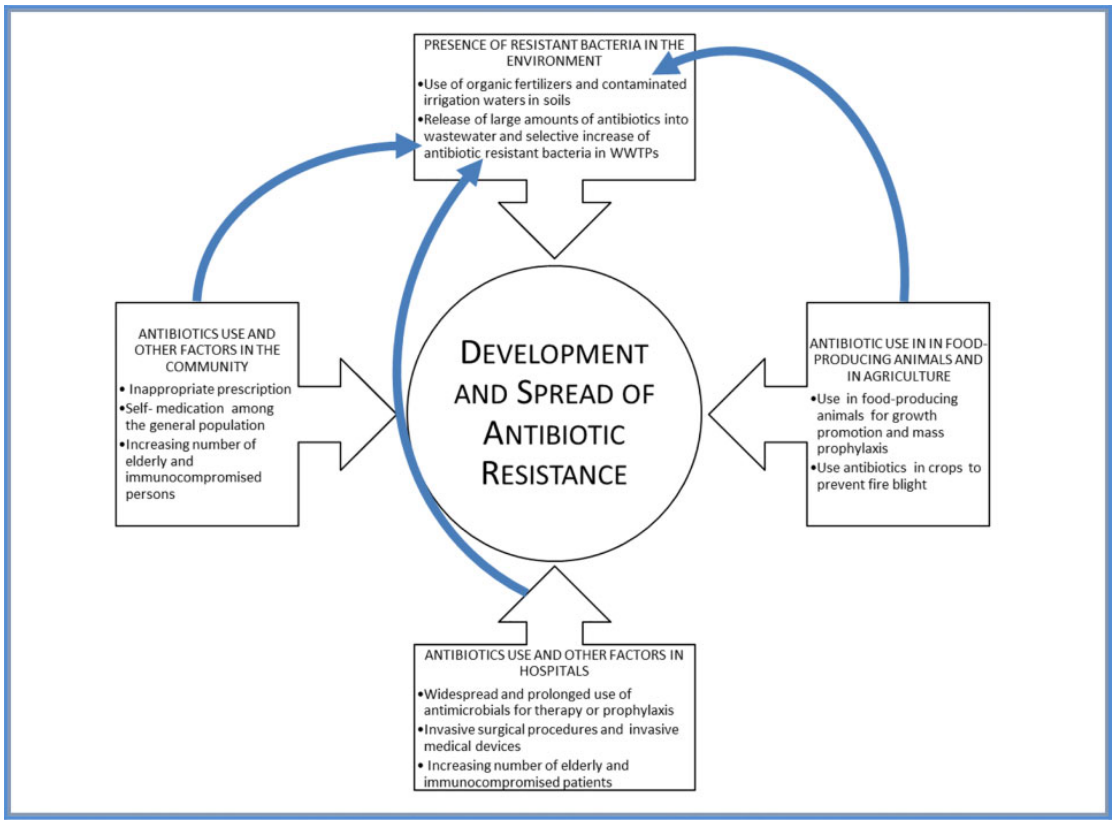

Figure 1.2 Factors involved in the spread of antibiotic resistance, in the sectors: human medicine in the community and in the hospital, animal production and agriculture, and the environment. These sectors are also connected among them: misuse of antibiotics in human beings, animals and agriculture is the main responsible for the presence of resistant bacteria in the environment. (Figure adapted from Prestinaci *et al*. (4) with permission).

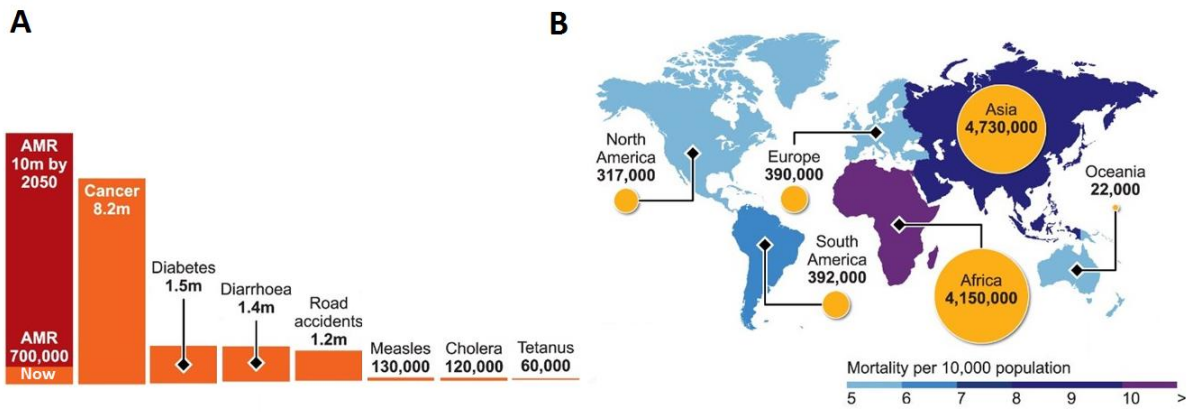

Figure 1.3 Worldwide death per year attributable to antimicrobial resistance (AMR) by 2050. **(A)** Antibiotic resistance versus other major causes of death anticipated in 2050. Antibiotic resistance will be the main cause of the death (10 million people dying every year). **(B)** Comparison of AMR between different areas of the world. (Figure adapted from Premanandh *et al*. (8) with permission).

1.2.1 *P. aeruginosa*

P. aeruginosa is an opportunistic Gram-negative bacillus that commonly infects immunocompromised and critically ill patients or those suffering from late stage malignant diseases (16-18). Notably, *P. aeruginosa* is primarily responsible of the life-threatening lung infection associated with CF (17, 19-21). Infections with MDR *P. aeruginosa* are usually associated with high morbidity and mortality rates (22, 23).

P. aeruginosa able to counteract the action of most of antimicrobial compounds either by its intrinsic, acquired or adaptive resistance mechanisms and in some cases it can acquire multiple resistance mechanisms (24). Because of the ability of MDR *P. aeruginosa* to evolve resistance towards most of clinically available antibiotics, it remains as a clinical challenge (17). For example, once chronic infection in the CF lung is established, MDR *P. aeruginosa* undergoes certain genetic modifications, including conversion from the nonmucoid to mucoid phenotype, downregulation of virulent factors and development of antibiotic resistance **(Figure 1.4)** (25-27). In CF lung infections the situation is more complicated, long term use of antibiotics in the treatment of *P. aeruginosa* infection in CF patients encourage the pathogen to become more resistant compare to non-CF infections (28). Intrinsically, *P. aeruginosa* gains the antibiotic resistance from its low permeable OM (about 1/100 of the permeability of *E. coli* OM), and from constitutively expressed membrane efflux pumps and secretion of β-lactamases (29-31). In addition, this pathogen can acquire resistance genes that alter the antibiotic binding sites and genes responsible for production of antibiotic inactivating enzymes (28, 32). Moreover, environmental stressors induce *P. aeruginosa* to develop an adaptive resistance for example formation of biofilm that protect the pathogen from surrounding environment in FC lung (28). Biofilm formation is the common feature of mucoid *P. aeruginosa*. Colonisation of bacteria within the biofilm increase their ability to adapt to the stressful lung environment, protects them from the immune system, and improves their tolerance to the antibiotics through reduction of bacterial metabolic activities and entry into a dormant persister state (25-27, 33, 34). In terms of pathogenesis, *P. aeruginosa* is seldom the cause an infection in healthy subjects. In most

cases, the underlying illnesses (e.g. disruption of physical barriers and immune deficiency diseases) are required to initiate the infection by this organism (35). *P. aeruginosa* pathogenicity is initially established by attachment and colonisation in the site of infection through the surface components (flagella and pili), and then invasion of eukaryotic cells by secreted factors (type III secretion proteins, extracellular products, QS molecules, and alginate) **(Figure 1.5)** (17, 36-38). It understood that *P. aeruginosa* undergoes extensive phenotypic and genotypic changes as the infection progress from acute to chronic phase; such modifications reflect the difference in pathogenicity between acute and chronic infections (39-41). Generally, isolates from chronic infection are less pathogenic compare to those from acute infection. Apparently, *P. aeruginosa* isolates in chronic infection loss their surface effectors (flagellum and pili) which are necessary for motility and adhesion and become avirulent in addition to the reduction of other extracellular virulent factors (24, 42). Most of the virulence factors associated with the acute and chronic *P. aeruginosa* infections are strongly driven by a QS, which is a complex cell-to-cell communication (43, 44). *P. aeruginosa* is able to communicate and establish its pathogenicity through three QS systems. The *Las* system which consists of a transcriptional regulator (LasR) and a synthase protein (LasI), the *Rh1* system which consists of two proteins RhlI and RhlR, and the third system is QscR which is significantly homolog to LasR and RhlR (45). These sensing systems are operated through tiny membrane-diffusible molecules known as autoinducers, these autoinducers are acyl homoserine lactones (AHLs) and function in a concentration-dependent manner. The concentration of these molecules is directly proportional to the bacterial concentration, when both concentrations increase to a critical point, the autoinducers become sufficient to induce the activation of specific genes resulting in an organised response among the whole bacterial population (45, 46). As QS systems play crucial roles in *P. aeruginosa* pathogenicity, targeting these machineries provides a promising antimicrobial strategy (45, 47-49). Anti-QS effects of different classes of antimicrobial compounds have been investigated in *P. aeruginosa* CF lung infections (50-56). Macrolides antibiotics typically Azithromycin (AZM), at concentrations much lower than the minimal

inhibitory concentration (MIC), effectively attenuate the activity of QS systems and inhibit the production of several virulence factors (e.g. proteases, DNase, lecithinase, and pyocyanin) (51, 53, 54, 56). In addition to AZM, the third-generation cephalosporin (ceftazidime) and the fluoroquinoline antibiotic (ciprofloxacin) were shown to significantly suppressed the expression of QS-mediated *P. aeruginosa* virulence at sub-inhibitory concentrations (50). Conversely, the sub-inhibitory concentration of the cationic antimicrobial peptide colistin extensively upregulated the biosynthesis of *P. aeruginosa* quinolone signal (PQS) genes (55).

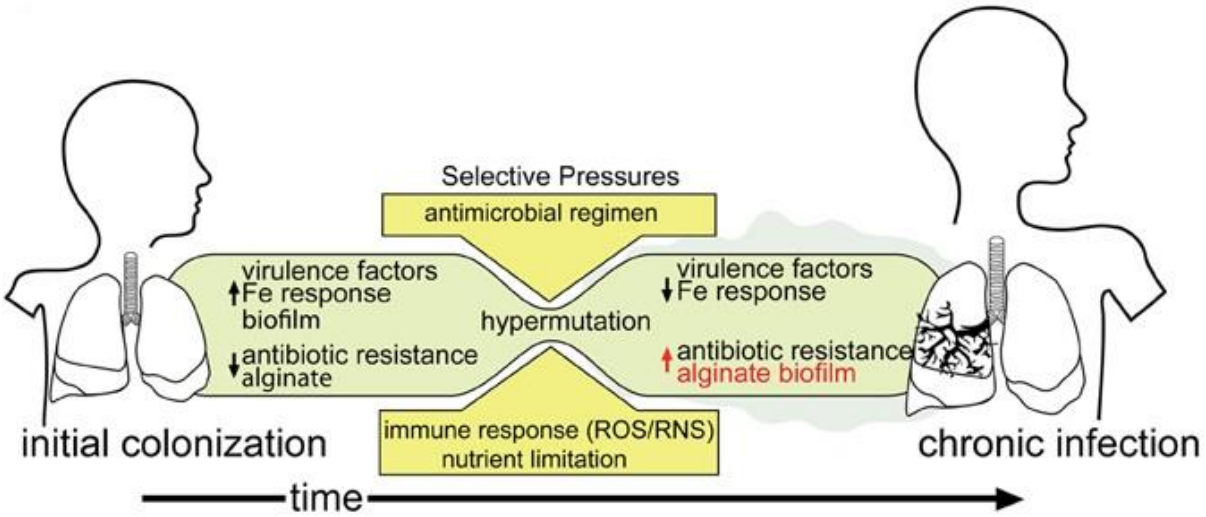

Figure 1.4 *P. aeruginosa* infection within a CF lung. As infection proceeds from an acute to the chronic state, phenotypes associated with chronic infection (e.g., mucoidy) become predominate, while acute virulence factors are downregulated. (Figure modified from Okkotsu *et al*. (27) with permission).

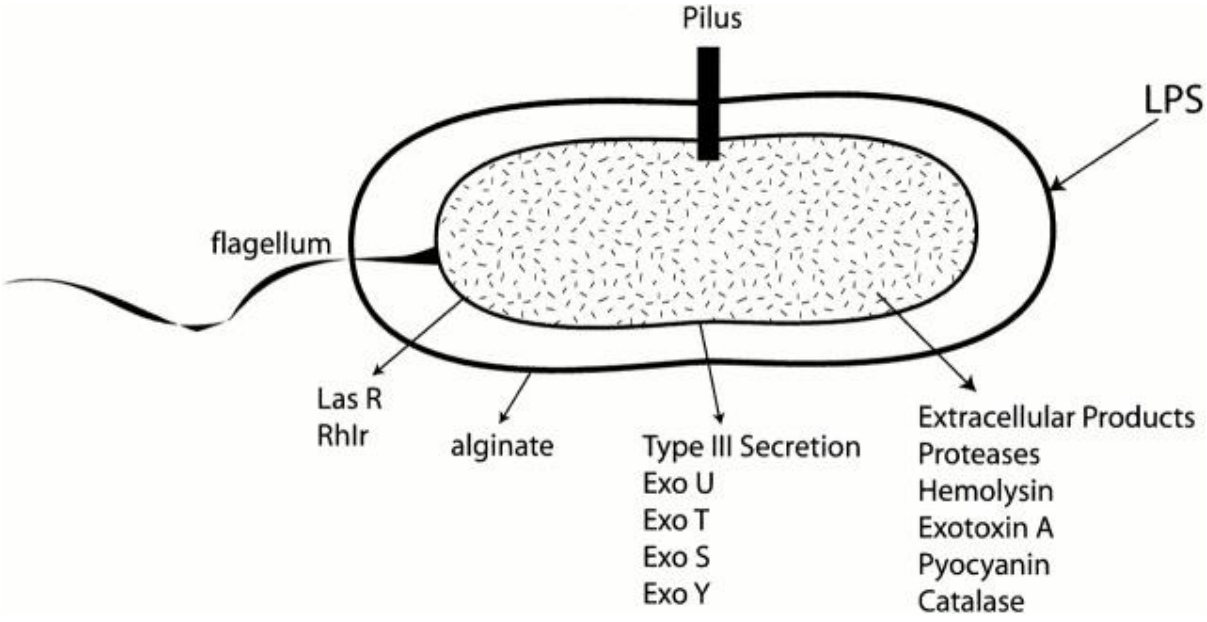

Figure 1.5 Virulence factors associate with *P. aeruginosa* infection: surface factors include flagellum, pilus and LPS. While, secreted factors, include extracellular products, quorum-sensing molecules, type III secretion proteins and alginate. (Figure adapted from Sadikot *et al.* (17) with permission).

1.2.2 *A. baumannii*

A. baumannii is an opportunistic, aerobic, non-motile, and non-fermenting coccobacillus Gram-negative that is clinically prevalent pathogen worldwide (57-59). Not unlike *P. aeruginosa*, MDR *A. baumannii* has been categorized as a "Serious Threat" by the CDC (12). In addition, WHO has grouped *A. baumannii* in priority 1 for targeting new antibiotic discovery (13). Beside their emerging antibiotic resistance, MDR *A. baumannii* species possess the capability to survive in different environmental settings particularly in the nosocomial setting, where antibiotic use is widespread (60-63). The probability of *A. baumannii* developing MDR is regrettably high among patients that undergo frequent and prolonged hospital stays, particularly solid organ transplant recipients due to them receiving of broad-spectrum antibiotics and immune suppression therapy (64, 65). It has been reported that *A. baumannii* establishes pathogenesis through a number of key factors including OmpA (a surface protein *syn.* Omp38), phospholipases (C and D), lipopolysaccharide (LPS), penicillin binding protein (PBP) and OMVs (59, 66, 67). Another important factor that contributes to *A. baumannii*'s virulence and survival in tough conditions is biofilm formation (68). Biofilms enable most *A. baumannii*

clinical isolates to adhere to the abiotic surfaces (e.g. urinary and venous catheters) which enhance their capability to cause nosocomial infections (69, 70).

Both the rapid development of innate resistance mechanisms and acquisition of external genetic determinants makes *A. baumannii* a formidable pathogen (57, 71). Recently, some of *A. baumannii* strains exhibit resistance to almost all clinically available antibacterial agents, apparently via large genomic islands containing clusters of antibiotic resistance genes interspersed with genetic vehicles including plasmids, transposons and integrons (71-82). Enzymatic degradation is another most prevailing mechanism of resistance developed probably by all MDR *A. baumannii* strains, this mechanism is mediated by many antibiotic-inactivating enzymes (e.g. β-lactamases including metallo-β-lactamases and serine oxacillinases, AmpC cephalosporinases, acetyltransferases, nucleotidyltransferases and phosphotransferases) (57, 83). In addition, MDR *A. baumannii* capable of developing a non-enzymatic resistance mechanism through alterations of antibiotic-targeting sites, modification of OM proteins, mutations of PBPs and overexpression of efflux pumps (57, 84-90). Resistance to the last line therapeutics such as the polymyxins has been increasingly reported in MDR *A. baumannii*, this resistance mechanism associated with mutations in the genes that responsible for biosynthesis pathway of lipid A or LPS loss (91-94). Moreover, some studies have shown that polymyxin-susceptible *A. baumannii* isolates display polymyxin hetroresistance when treated with polymyxin due to the presence of subpopulation (95-98).

1.2.3 *K. pneumoniae*

K. pneumoniae, an Enterobacteriaceae family member, is non-motile, encapsulated, lactose-fermenting, facultative anaerobic Gram-negative coccobacilli (99). Typically, *K. pneumoniae* is an opportunistic organism responsible for a high proportion of nosocomial infections including pneumonia, urinary tract infections, bloodstream infections, and soft tissue infections particularly in immunocompromised patients (100, 101). *K. pneumoniae* carbapenemase (KPC) and New Delhi metallo-β-Lactamase (NDM) producing *K. pneumoniae*, members of

Carbapenem-resistant *Enterobacteriaceae* (CRE), are resistant to the carbapenems (a class of β-lactam antibiotics) which have extended spectrum of activity compared to penicillins and cephalosporins (102). Some *K. pneumoniae* isolates produce extended-spectrum β-lactamase (ESBL) enzymes which enable these species to resist penicillins and cephalosporins but remain susceptible to carbapenems (103). Therefore, the CDC has categorised KPC- and NDM-producing *K. pneumoniae* as an urgent threat, while ESBL- producing *K. pneumoniae* as serious level of threat (12). And according to WHO report in 2017, carbapenemase-producing *K. pneumoniae* isolates are listed in the critical priority for new antibiotics discovery and development (13). Hospital-acquired *K. pneumoniae*'s infections appear to be chronic due to their ability to form biofilm that protects the bacteria from human defence mechanisms and antibiotics activity (104); and the presence of phenotypic MDR *K. pneumoniae* that produce ESBL and carbapenemase enzymes (99, 105, 106). The virulence of *K. pneumoniae* evoked through a number of determinants including production of capsule polysaccharide (CPS), LPS, fimbriae, OM-derived proteins, iron acquisition and utilization of nitrogen (99). Among these virulence factors, the CPS is the most important one and it is the characteristic feature of the hyper-virulent *K. pneumoniae*, it composed of an acidic polysaccharide and responsible for the hypermucoviscosity and colonisation of the invasive hyper-virulent *K. pneumoniae* (107, 108). The hyper-virulent *K. pneumoniae* isolates produce more than 78 capsular or K antigen serotypes particularly K1 and K2 serotypes (109-111). The severity of the invasive infection caused by hyper-virulent *K. pneumoniae* is directly proportional to the degree of hypermucoviscosity (112). The CPS protects the hyper-virulent *K. pneumoniae* from host defences by suppressing the host immune responses, increasing the resistance to the phagocytosis, suppressing the inflammatory processes, augmenting the resistance to the polycationic antimicrobial peptides and impairing the maturation of dendritic cells (DCs) (113-119). Unlike other Gram-negative bacilli, life-threatening invasive infections caused by hypermucoviscous *K. pneumoniae* can be metastatic (i.e. spread to other organs) in some cases such as pyogenic liver abscess even in the presence of host defence mechanisms (120). In

addition to the production of β-lactamases, formation of biofilm and overproduction of CPS, MDR *K. pneumoniae* isolates can establish AMR through several other mechanisms including production of other enzymes such as macrolide esterases that inactivate macrolide antibiotics (121), and 16S rRNA methyltransferases which alter aminoglycosides binding sites (122); and overexpression of efflux pumps that render many antibiotic classes ineffective (123). Although colistin (polymyxin E) and polymyxin B are resorted as the last therapeutic option for the treatment of carbapenem-resistant *K. pneumoniae* (CRKP), surveillance reports have shown 17% and 43% resistance rate to colistin caused by CRKP in Taiwan and Italy respectively (124, 125). Therapeutically ESBL-producing *K. pneumoniae* isolates appear to be susceptible to Carbapenems, aminoglycosides, quinolones, third- and fourth-generation cephalosporins, and to combination of β-lactams/β-lactamase inhibitors (126). However, CRKP isolates, and due to the presence of plasmid mediated cross-resistance, are resistant to almost all antibiotic classes including all β-lactams even in the combination with β-lactamase inhibitors (127, 128). Tigecycline, tetracyclines, polymyxins and aminoglycosides are effective against infections caused by CRKP (129). Very recently, two novel combinations of lactam/β-lactamase (Ceftolozane/tazobactam and ceftazidime/avibactam) have been approved clinically for the treatment of serious infections evoked by both ESBL-producing *K. pneumoniae* and CRKP (130).

1.3 Polymyxins: an overview

Polymyxins are cationic antimicrobial polypeptides firstly isolated from *Paenibacillus polymyxa*, a Gram-positive soil bacterium, in 1947 (131, 132), and approved for clinical use in the late 1950s (133). The use of these antibiotics was declined rapidly due to the reported side-effects (particularly nephro- and neuro-toxicity) and the availability of safer antibiotic (e.g. β-lactams and aminoglycosides); therefore, their clinical application waned in the 1970s (134). However, in 1990s and due to emerge of MDR Gram-negative bacteria, the use of polymyxins have been restored and they preserved as the last therapeutic option for these problematic

pathogens (135). Moreover, the re-establishment of polymyxins is largely due to their good anti-Gram negative activity (136).

1.3.1 Chemistry of polymyxins

Polymyxins are lipopeptides that are amphipathic in nature (137). The pharmacological properties of polymyxins depend fundamentally on their physicochemical properties (15). The core structure of polymyxins composed of a polycationic peptide made up of 10 amino acids that is attached to *N*-terminal fatty acyl chain **(Figure 1.6)**. These lipopeptides are characterised by the presence of both a cyclic heptapeptide ring and a linear tripeptide segment attached to the *N*-terminal fatty acyl chain (138, 139). Although polymyxins (A, B, C, D and E) were originally discovered, only polymyxin B and colistin (polymyxin E) have been approved for clinical application (137). Commercial preparations of polymyxin B and colistin used clinically consists of a diverse mixture of structurally related congeners that differ only in the substitution at *N*-terminus of their fatty acyl group and position 6 and 7 amino acids (138, 140). The only difference between polymyxin B and colistin is in replacement of D-phenylalanine in polymyxin B at position 6 with D-leucine in colistin (141, 142). Polymyxin B1 (6-methyl-octanoyl fatty acyl) and polymyxin B2 (6-methyl-heptanoyl fatty acyl) are the main components of polymyxin B, while colistin A (6-methyl-octanoyl fatty acyl) and colistin B (6-methyl-heptanoyl fatty acyl) are the main components of colistin (138, 140). The actual proportions of the components of both polymyxin B and colistin are highly variable between different manufacturers and even between patches of the same manufacturer (143, 144). Both of polymyxin B and colistin are available in distinctly different commercial formulation, polymyxin B is administered parenterally in its active sulphate salt form. While, colistin is commercially formulated as inactive sulfomethylated pro-drug known as colistimethate sodium (CMS) (145, 146).

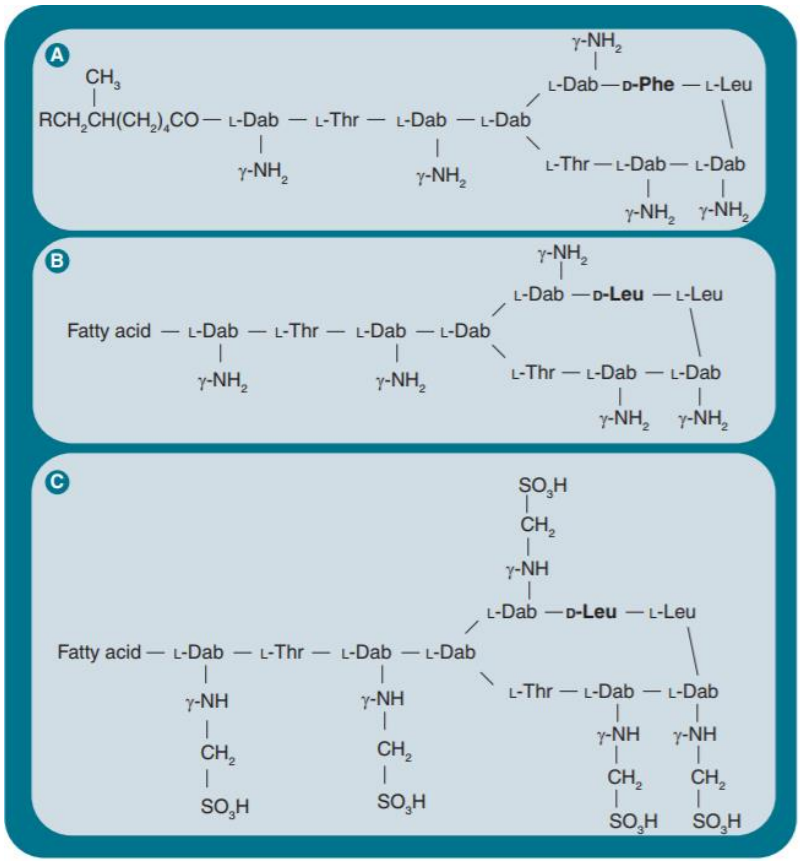

Figure 1.6 Chemical Structure of polymyxin B, colistin and colistin prodrug (colistimethate). **(A)** Structures of polymyxin B1 and B2. **(B)** Structures of colistin A and B. **(C)** Structures of colistimethate A and B. Fatty acid: 6-methyloctanoic acid for colistin A and colistimethate A and 6-methylheptanoic acid for colistin B and colistimethate B. Dab: diaminobutyric acid; Leu: Leucine; Thr: Threonine; and γ is the amino group involved in the linkage. (Figure adapted from Kwa *et al*. (141) with permission).

1.3.2 Mechanism of action of polymyxins

Due to the structural similarities between polymyxin B and colistin, both of them exert identical mechanism of antimicrobial activity and narrow spectrum of activity against Gram-negative bacteria (131, 147). Although the researchers have suggested many models to explain the mechanism of action of polymyxins, the precise bactericidal mode of action of these lipopeptides is not fully understood (148-153). What is well know is that the bacterial OM (particularly the LPS component) is the principal target of polymyxins (15, 154, 155). At this point, a detailed overview of the architecture of the Gram-negative bacterial OM is requisite for better appreciation of the putative mode of action of polymyxins (15, 155). Structural analysis of Gram-negative bacterial cell wall revealed that it composed of two membranous layers, an

asymmetric OM and a symmetric inner membrane **(Figure 1.7A)** (156). Further analysis showed that the OM built up of outer leaflet (predominantly made up of LPS) and inner leaflet (predominantly made up of glycerophospholipids) (156, 157). Basically, LPS consist of repeated sugar residues which made up the variable *O*-antigen, the rigid oligosaccharide moiety and lipid A anchor (156). Polymyxins are initially target lipid A part of LPS (15, 158). Structurally, lipid A is composed of β-1′-6-linked D-glucosamine (GlcN) disaccharide, which is enzymatically phosphorylated at the 1- and 4’ positions and acylated with six acyl chains **(Figure 1.7B)**. Four of the six acyl chains are β-hydroxyacyl chains, which are attached directly to the glucosamine sugars and usually 12 to 14 carbons in length, the other two chains are attached to a β-hydroxyl group (155). Magnesium and Calcium divalent cations are responsible for bridging the neighboring LPS molecules (15). The complex quasi-crystalline nature of LPS monolayer together with its very low hydrophilicity (due to the hydrophobic lipid A anchor) contribute to the efficient permeability barrier property of Gram-negative bacterial OM against detrimental substances (159).

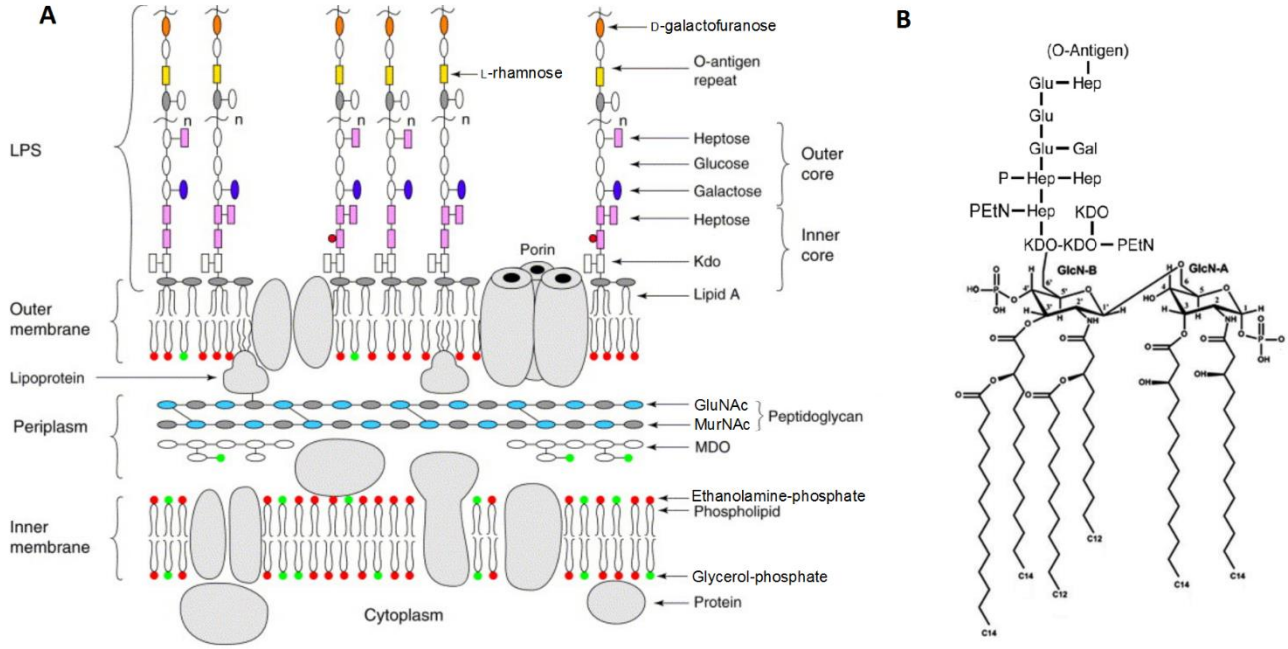

Figure 1.7 Gram-negative bacterial cell envelope. **(A)** Membrane layers of the cell envelope, which composed of OM and inner membrane. The OM composed of outer asymmetric leaflet (predominantly LPS) and inner symmetric leaflet of phospholipids (Figure modified from Wyckoff *et al*. (160)). **(B)** The structure of the lipid A moiety. The lipid A part of LPS composed of β-1′-6-linked disaccharide of glucosamine. (Figure modified from Velkov *et al*. (155) with permission). Abbreviations: Kdo, 3-deoxy-

D-manno-octulosonic acid; LPS, lipopolysaccharide; MDO, membrane-derived oligosaccharides; GluNAc, N-acetylglucosamine; MurNAc, N-acetylmuramic acid.

Biophysical studies have led to the proposition of a number of models for the bactericidal action of polymyxins, however, the 'self-promoted uptake' hypothesis is the most accepted mechanism of action **(Figure 1.8)** (15). Generally, it has been proposed that polymyxins bind to the Gram-negative bacterial OM and result in perturbation of the membrane integrity(161). In deep, due to their amphipathic nature, polymyxins initially interact with a specific OM binding site (the lipid A moiety) via electrostatic attraction followed by hydrophobic interaction with the OM leaflet, the results are membrane ultrastructural perturbations, osmotic imbalance, and consequently bacterial cell death (150, 151, 154). The electrostatic attraction occurs between the cationic diaminobutyric acid (Dab) residues of polymyxins and the anionic phosphate groups of lipid A, with subsequent competitive displacement of LPS stabilizing cations (Ca^{2+} and Mg^{2+}). This interaction facilitates the insertion of hydrophobic N-terminal fatty acyl chain and amino acid residues at position 6 and 7 into the outer leaflet (137, 162). Moreover, it has been suggested that polymyxins incorporate into the inner leaflet and cytoplasmic inner membrane resulting in destruction of membrane phospholipids and forming "pore-like aggregates" which increase the permeability of bacterial membrane and ultimately cell death (15, 162, 163).

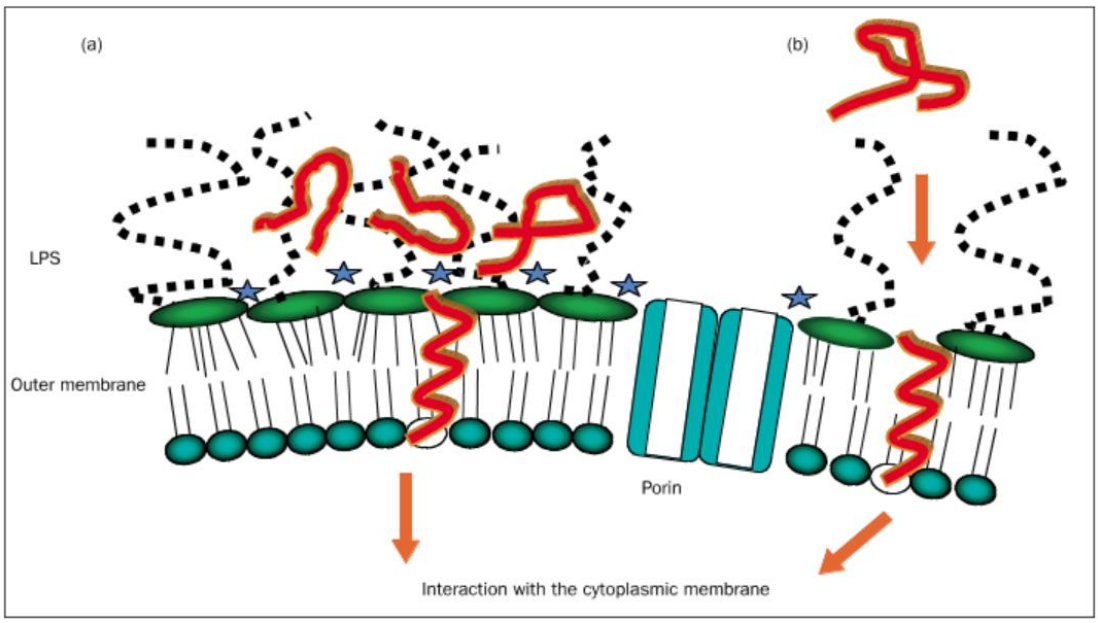

Figure 1.8 The 'self-promoted uptake' pathway for plymyxin B interactions with lipid A. **(A)** The binding of polymyxin B to the divalent cationic binding site of LPS, and disrupting the membrane **(B)** Electrostatic interaction of polymyxin B and anionic surface of cytoplasmic membrane. (Figure adapted from Hancock REW,(163) with permission).

Binding studies demonstrated that polymyxins have a binding affinity to 16S A-site of *E. coli* ribosomes, however, no translational changes have been noticed (164). Schindler *et al*. showed that polymyxin treatment of *Salmonella typhimurium* and *E.coli* results in production of membranous blebs (i.e. OM projections) and aggregation of nuclear material due to the significant accumulation of polymyxin within the bacterial cytoplasm (165). Moreover, cell division is another process that is significantly affected by polymyxins treatment (166). Further investigations have demonstrated that polymyxins could induce cell death via generation of reactive oxygen species (ROS) including superoxide, hydrogen peroxide, and hydroxyl radicals. Collectively, these ROS induce oxidative damage of DNA, lipid, and Fe-S dependent proteins inhibit respiratory enzymes such as NADH-quinone oxidoreductase, NADH cytochrome c and NADH dehydrogenase; and ultimately cause cell death (149, 167-169). However, bacterial death associated with polymyxin-induced ROS are still a subject of debate (170-172).

1.3.3 Mechanisms of polymyxin resistance

The emergence of polymyxins resistance has been increased dramatically during the last decades due to the improper global use of polymyxins. The modification of outer membrane LPS, particularly lipid A domain, is the main encountered mechanism of polymyxin resistance in Gram-negative pathogens (173). The remodelling of lipid A has been observed in *E. coli, P. aeruginosa*, *A. baumannii K. pneumoniae*, and *Salmonella enterica* serovar Typhimurium (91, 93). This lipid A remodelling is triggered by enzymatic addition of either cationic sugar moiety (4-amino-4-deoxy-L-arabinose), phosphoethanolamine, or galactosamine resulting in suppression of LPS affinity to polymyxins (91-93, 173, 174). In addition, polymyxins, as a membrane stressor, induce Gram-negative pathogens to fortify their OM by hepta-acylation of lipid A through the addition of palmitate at the R-2-hydroxymyristate of lipid A, the resulting hepta-acylated OM becomes less permeable to polymyxins (175, 176). Surprisingly, some lipid A deficient mutants of *Neisseria meningitidis* and *A. baumannii* have been demonstrated to be viable in spite of complete lack of LPS; therefore these mutants are naturally resistant to polymyxins (94, 177). These resistance mechanisms to polymyxins are driven by dual component-component regulatory systems; however, the detailed molecular mechanism of resistance remains obscure due to the complexity of these regulatory systems (178). To date, about 143 two-component regulatory systems have been demonstrated in *P. aeruginosa* (179). Only PmrA/PmrB, PhoP/PhoQ, ParR/ParS, ColR/ColS and CprR/CprS systems are studied extensively due to their direct association with antibiotics (predominantly polymyxins) resistance (178, 180-185). The PhoPQ and PmrAB systems induce the modulation of *arnBCADTEF* LPS modification operon under Mg^{2+} starvation which in turn induce the insertion of 4-aminoarabinose sugar to lipid A that leads to the reduction in LPS negative charge and ultimately reduce the polymyxin-LPS binding affinity (178). Contradictorily to the above mentioned Mg^{2+}-dependent regulatory systems (PhoPQ and PmrAB), Fernandez *et al*. have demonstrated a novel Mg^{2+}-independent two-regulatory system (ParR-ParS) which is directly induced by cationic antimicrobial peptides (interestingly polymyxins). This regulatory system

extensively participates in the development of polymyxins adaptive resistance without contribution in the development of intrinsic resistance (184). In another study, Muller *et al*. revealed that, in addition to its role in the adaptive resistance to polymyxins through LPS modification, ParR-ParS system play an essential role in adaptation of *P. aeruginosa* to other different antibiotic classes such as aminoglycosides, fluoroquinolones, and β-lactams via distinctly different mechanisms of resistance (i.e. upregulation of efflux pump, and reduction of porin pathway) (186). Moreover, it has been shown that ParRS system of *P. aeruginosa* controls the regulation of both the efflux pump and the QS system in the absence of antimicrobial compounds (187). Consistently, Fernandez *et al*. have found that *P. aeruginosa* developed adaptive resistance to polymyins through the induction of cationic peptide resistance two-component system (CprRS) independently of the ParRS system (188).

Away from LPS modification, some Gram-negative bacteria exhibit resistance to polymyxins by other mechanisms, for example, *P. aeruginosa and K. pneumoniae* resist polymyxins by overexpression and production of anionic CPS which protects the bacterial OM from polymyxins and other antimicrobial peptides (117). Moreover, overproduction of OM efflux pump and biofilm formation are commonly linked to polymyxins and other antibiotics resistance (189). Besides that, Gram-negative bacteria produce OMVs which act as a decoy for polymyxins and other membrane stressors and thus protect the bacterial cell from their killing action (190). More recently, Liu *et al*. have reported for the first time a plasmid-incorporated MCR-1 gene in *E. coli* which is responsible for plasmid-mediated polymyxins resistance (191). Since that time, this new mechanism of polymyxins resistance has subjected to extensive studies in both human and animal models (192-194). The MCR-1 gene belongs to the family of phosphoethanolamine transferases; and it participates in the modification of bacterial LPS by incorporating phosphatidylethanolamine into lipid A moiety of the LPS (191, 195). Due to the aforementioned polymyxins resistance mechanisms, the urgent global action is needed at least to minimise the resistant to these last therapeutic resorts by MDR pathogens.

Table 1.1 Polymyxins resistance mechanisms in MDR Gram-negative bacteria. The table adaptive from Olaitan *et al.* (196) with permission.

Resistance mechanism	Genes involved	Bacteria
Modification of the lipid A or Kdo with aminoarabinose	*arnBCADTEF* operon and *pmrE*	*Salmonella enterica, K. pneumoniae, E. coli, P. aeruginosa* and *Burkholderia cepacia complex*
Modification of the lipid A with phosphoethanolamine	*pmrC*	*S. enterica, K. pneumoniae, E. coli* and *A. baumannii*
Activation of LPS-modifying operon by mutations in TCSs	*pmrA/pmrB* and *or phoP/phoQ*	*S. enterica, K. pneumoniae, P. aeruginosa* and *A. baumannii*
Inactivation of phoP/phoQ negative feedback regulator	*mgrB*	*K. pneumoniae*
Modification of the Kdo with phosphoethanolamine	*eptB, phoP/phoQ* and *mgrR*	*E. coli*
Increased acylation of lipid A enhancing its modification with aminoarabinose	*lpxM*	*S. enterica, K. pneumoniae* and *E. coli*
Trapping of polymyxins by capsule		*K. pneumoniae and P. aeruginosa*
Efflux pump	*acrAB* and *kpnEF*	*K. pneumoniae*
Loss of LPS	*lpxA, lpxC* and *lpxD*	*A. baumannii*
Glycosylation of lipid A with hexosamine		*A. baumannii*
Acquired/adaptive resistance to polymyxins through LPS modification with aminoarabinose	*colR/colS, cprR/cprS* and *parR/parS*	*P. aeruginosa*
Overexpression of outer membrane protein OprH	*oprH*	*P. aeruginosa*

1.3.4 Pharmacokinetics and pharmacodynamics (PK/PD) of polymyxin B and colistin

Commercially, colistin is available either as colistin sulphate (mainly for topical application) or as an inactive prodrug of sodium salts of colistin methanesulfonate (CMS) (generally for parenteral use). For inhalation use, both forms are convenient. In contrast, polymyxin B is used parenterally in its active sulfate salt (145, 197, 198). Chemically, CMS is formulated by hiding the primary amines of the Dab residues with methanesulfonate groups which carry negative charges at physiological pH (146). Our group has performed diligent PK/PD studies of

polymyxin B and colistin in an effort to optimise their clinical use (199-201). Although Polymyxin B and colistin are structurally similar to each other and excrete the same *in vitro* antimicrobial actions however *in vivo,* they exhibit remarkably different PK profiles (198). Polymyxins exhibit negligible oral and dermal bioavailability due to their large particle size, therefore, they are administered parenterally (intravenously and intramuscularly) for serious systemic infections or by nebulization for pulmonary infections (133, 162, 197, 198).

Unlike colistin, CMS is unstable and inactive neither *in vitro* nor *in vivo*; and it is slowly converted to colistin and a number of methanesulphonate derivatives after parenteral administration. Another PK difference is that CMS mainly undergoes renal excretion, while colistin is excreted predominantly via other routes **(Figure 1.9)** (133). Zavascki *et al*. and Abdelraouf *et al*. have reported that polymyxin B excreted renally in less than 1% of its administered dose **(Figure 1.9)** (202, 203).

Time-kill studies have shown that polymyxins (polymyxin B and colistin) exert their rapid antibacterial activity against Gram-negative bacteria in a concentration-dependent manner; however, the bacterial growth recovery occurs rapidly (199-201, 204). It is very likely that resistance to polymyxin could quickly emerge due to the existence of polymyxin-resistance subpopulation (heteroresistance) within the polymyxin-susceptible colony. This heteroresistance possibly results from prolong polymyxins therapy therefore proper monitoring of the resistance during polymyxin treatment is guaranteed (95, 96, 205).

Although, some animal studies have shown that antibacterial activity of polymyxins can be measured by calculating the ratio of the area under unbound plasma concentration-time curve to MIC (*f*AUC/MIC) which is the PK/PD index. However, these data are clinically inapplicable due to the shortage of the information on the unbound plasma concentration of polymyxins in patients (206, 207).

Garonzik *et al*. have suggested that CMS/colistin combination might be the best approach to achieve the desired plasma colistin concentration instead of CMS monotherapy which is unable to provide adequate colistin concentration in the plasma, particularly when the MIC of the

infecting bacteria is >0.5 mg/L and the creatinine clearance is >70 ml/min/1.73 m^2 (208). In this study, CMS was administered daily by IV infusion to a total of 105 patients in a dose range of 200 mg colistin base activity (CBA) which equals to about 6.666 million units (MU) of CMS, the average steady-state plasma concentration of formed colistin was 0.48 – 9.38 mg/L (median: 2.36 mg/L) **(Figure 1.10)**. The inadequate concentration of formed colistin in the plasma is due to the slow biotransformation of CMS to colistin, therefore considering of a loading dose and long dose interval is vital to achieve the steady-state plasma concentration of formed colistin rapidly (209). In contrast to CMS, a clinical study conducted on 24 critically ill patients had revealed that the PK of polymyxin B is profoundly affected by total body weight and that kidney function does not affect the required polymyxin B daily dose and the total body clearance (210). To date limited PK/PD data are available for all clinically used polymyxins, therefore more data about polymyxins PK/PD from clinical studies are urgently needed to optimise their dosage regimen particularly in critically ill patients.

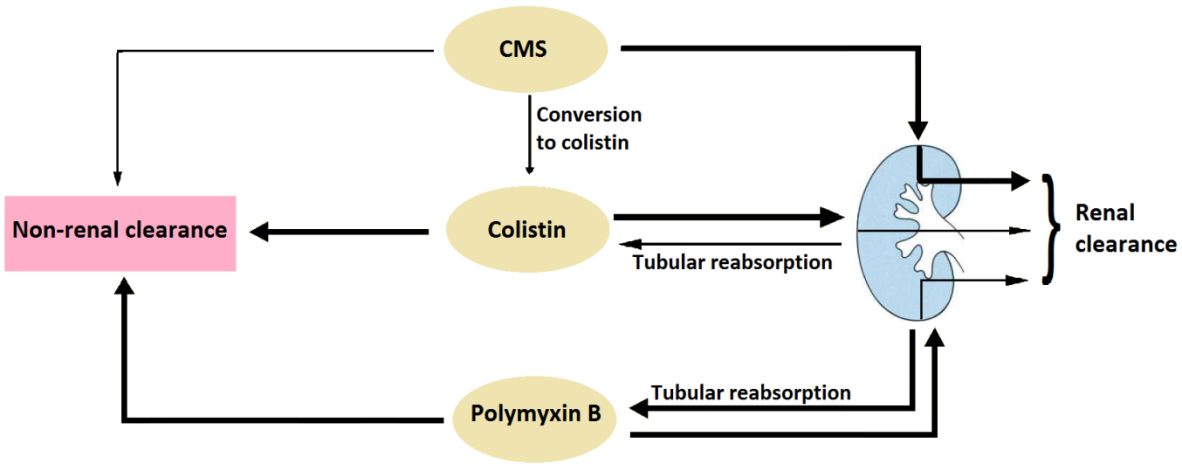

Figure 1.9 Illustration of polymyxins clearance pathways. The relative thickness of the arrows represents the degree of renal and non-renal clearance of the three clinically available polymyxins (CMS, colistin and polymyxin B) in individual with healthy kidneys. (Figure modified from Nation *et al*. (146) with permission).

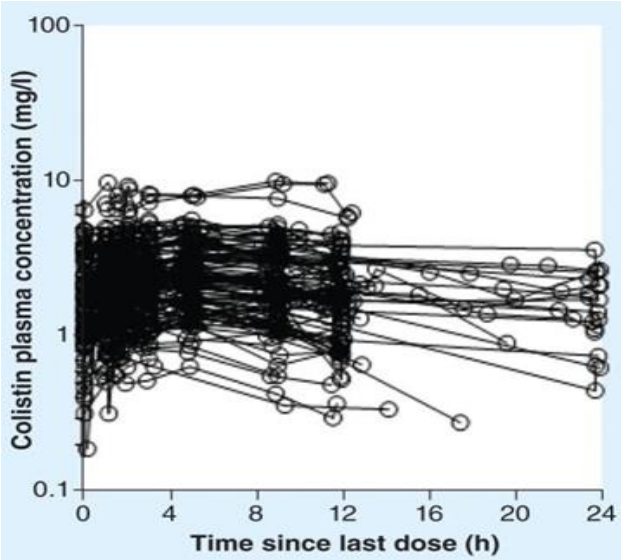

Figure 1.10 Steady-state plasma concentration-time profiles of CMS-derived colistin. The data obtained from 105 critically ill patients including 89 not on renal replacement, 12 on intermittent haemodialysis, and 4 on continuous renal replacement therapy. The administered daily dose of CMS was in a range of 200 mg CBA. (Figure adapted from Garonzik et al. (208) with permission).

1.3.5 Toxicity: nephrotoxicity and neurotoxicity

Clinically, polymyxins-induced nephrotoxicity and neurotoxicity have been reported following parenteral administration of polymyxins in several studies conducted during the 1960s and 1970s (211-214). Nephrotoxicity of parenterally administered polymyxins is the most common and essential dose-limiting adverse effect, and it was the cause of their early replacement by other antibiotics which were considered 'less' toxic (e.g., gentamicin and second- and third-generation cephalosporins) (197, 215, 216). Koch-Weser *et al*. have reported that during 317 courses of IV CMS conducted on 288 patients, nephrotoxicity and neurotoxicity occurred in 20.2% and 7.3% respectively (212). The administration of polymyxin B in higher intramuscular doses and the existence of dibucaine hydrochloride in the intramuscular formulation of CMS are the significant causes of polymyxins-induced nephrotoxicity and neurotoxicity (134). Recent comparative studies found that the rate of nephrotoxicity is higher with CMS/colistin than that observed with polymyxin B (possibly due to the intracranial conversion from CMS and partially methanesulfonate derivatives) (217, 218). Moreover, Falagas *et al*. had suggested that the frequency and severity of polymyxins-induced toxicities are less than what has been reported in early investigations, these observations might be due to the fact that the

intramuscularly administered CMS in recent studies was used intravenously in old reviews (134). Recently, studies conducted on patients in intensive care unit (ICU) demonstrated that about 14.3%-18.6% of colistin-treated patients experienced impaired renal function (219, 220). In spite of their toxicities, polymyxins proved to be safer than other treatments. Garnacho-Montero *et al*. have performed a comparative study on patients with ventilator-associated pneumonia evoked by *A. baumannii*, the renal failure observed in 24% of patients treated with IV CMS compare to 42% of patients received IV imipenem/cilastatin (221). In a clinical study, Hartzell *et al*. had revealed that although CMS therapy associated with mild renal dysfunction, however the permanent renal damage and the need for renal replacement therapy or cessation of the treatment are not necessarily occurred (222). The most common clinical manifestations of polymyxin-induced renal insufficiency are the elevation of serum creatinine level and reduction in creatinine clearance, haematuria, proteinuria, cylindruria, or oliguria (139), in addition to the development of acute tubular necrosis in some cases (223). It has been suggested that the nephrotoxic effect of polymyxins is concentration-dependent and includes loss of membrane integrity, which permits the entry of ions and water resulting in cell rupture (224, 225). Moreover, colistin had been demonstrated to be an essential promoter of the trans-epithelial conductance of the urinary bladder epithelium (226). Polymyxin-induced nephrotoxicity could be exacerbated by co-administration of other nephrotoxic compounds (e.g., diuretics) (212, 227). Furthermore, early clinical studies had reported that the incidence of polymyxin-induced neurotoxicity was dose-dependent and less than that of nephrotoxicity. Predominantly, paraesthesia and respiratory apnoea are the most common polymyxin-induced neurotoxic effects (212, 228). In contrast, studies over the past two decades demonstrated that there is no association of neuromuscular blockade or apnoea with polymyxins treatment (219, 220, 229). Polymyxins inhibit the release of acetylcholine to the synaptic cleft by interfering with the presynaptic receptors, resulting in neuromuscular blockage (230). Another proposed mechanism of polymyxin-induced neurotoxicity suggested that polymyxins competitively and shortly inhibit acetylcholine release followed by prolonged depolarisation phase which is

accompanied by depletion of calcium ion (231). The neurotoxic effects of polymyxins could be aggravated by several factors such as hypoxia, renal impairment, myasthenia gravis and concomitant administration of polymyxins with muscle-relaxants, narcotics, sedatives, anaesthetic drugs, or corticosteroids (232-234).

1.3.6 Polymyxins combination therapy

Evidence from clinical studies revealed that the combination of antibiotics is a promising approach to eradicate MDR Gram-negative bacteria and significantly lower the mortality rate compared to monotherapy (235-237). In many observational studies that had been conducted in intensive care unit (ICU) on patients with bacteraemia, surgical site infections, pneumonia, and septic shock, about 25% - 50% of the patients have been subjected to a combination of antimicrobial therapy (238). Hilf *et al*., in a multicentre comparative study, have reported that the combination of antibiotics significantly reduced the mortality rate compared to antibiotic monotherapy (27% versus 47%) in patients with *P. aeruginosa* bacteraemia (239). Another retrospective clinical study of malignancies-associated *P. aeruginosa* bacteraemia, the cure rates, which "defined as eradication of all signs and symptoms of pseudomonal infections", were 72% and 29% for both combination and monotherapy respectively (240). On the contrary, in other comparative studies, there was no difference in mortality rate or cure rate between the combination and the monotherapy against MDR Gram-negative bacteria (241-244). Therefore, the clinical efficacy and safety of antimicrobial combination therapy are still needed to be carefully evaluated particularly in term of the adverse toxic effects, emerge of resistance and the cost of the treatment course (238).

Due to the rapidly emerged polymyxin resistance and high mortality rate (47-67%) accompanying polymyxin monotherapy, therefore the use of these antibiotics as monotherapy is a non-optimal solution to eradicate MDR Gram-negative pathogens (245-247). Combination of polymyxins with other antimicrobial compounds is an alternative strategy to polymyxin single therapy, and such technique becomes increasingly utilised in clinical situations (248).

Interestingly, combining polymyxins with antibiotics from other classes demonstrates synergistic activity against most of the problematic MDR Gram-negative pathogens; and gives a chance to reduce the concentration of each compound which in turn decreases the probability of the occurrence of adverse effects (162, 249). *In vitro* analysis showed that colistin plus carbapenems (particularly doripenem) synergistically kill MDR *P. aeruginosa*, *K. pneumoniae* and *A. baumannii* (250-252). Clinically, the combination therapies (colistin + tigecycline and colistin + tigecycline + meropenem) against bloodstream infections caused by KPC- producing *K. pneumoniae* resulted in significantly higher survival rate (69.7% and 87.5% respectively) compare to 45.7% survival rate with colistin or tigecycline monotherapy (253). *In vitro* studies conducted by Zusman *et al*. and Ly *et al*. demonstrated that polymyxins and carbapenems combinational therapy against *A. baumannii, P. aeruginosa* and *K. pneumoniae* significantly reduce the development of polymyxin resistance (254, 255). Similarly, *in vitro* studies showed that polymyxins and rifampicin combinations exerted a synergistic antimicrobial activity against MDR Gram-negative pathogens (256-258). However, Durante-Mangoni *et al*. and Pogue *et al*. in multi-centre, randomised clinical studies have revealed that there was no difference in mortality rate between colistin monotherapy and the combination of colistin and rifampicin in critically ill patients with XDR *A. baumannii* (259, 260). Despite the *in vitro* synergistic activity of the combination of polymyxins and other antibiotics, clinical observations have shown that polymyxins and other antibiotics co-therapy could significantly improve the microbiological clearance rate but showed no difference in improvement of clinical outcome compare to polymyxins alone (259-263). The possible explanation of this lacking in the clinical cure is the shortage in available PK/PD data of the combination therapy and the inadequate translation of the *in vitro* synergies into proper clinical outcomes; therefore further clinical studies to evaluate the PK/PD and toxicity parameters are highly guaranteed (260, 261).

1.4 Outer membrane vesicles (OMVs): an overview

Microorganisms particularly Gram-negative bacteria constitutively secrete spherical bi-layered membranous structures, which are ranging between 20 and 200 nm in diameter, collectively named Outer membrane vesicles (OMVs) (157, 264). Historically, these OMVs have been reported for the first time in *Vibrio cholera* bacteria-free filtrate in 1959 (265). Later in 1965 and 1966, these vesicles were also reported in cultures of *E. coli* after growing in media contains less lysine and they were described as LPS-rich globe-shaped "bags" (266, 267). Later, it has been found that almost all Gram-negative pathogens secrete these membrane-associated spherical structures; therefore they were called 'membrane vesicles', 'extracellular vesicles', 'outer membrane fragments' or 'blebs' (268-272). To date, the most acceptable historical name is the outer membrane vesicles (273). Structurally, these nanoparticles composed of lipids and proteins derived mainly from the OM and periplasm (274). The LPS and phospholipids (such as glycerophospholipids, sphingolipids and glycerolipids) are the principal lipid contents of the OMVs. Additionally, several other studies showed that OMVs contain proteins derived from cytoplasmic membrane and cytoplasm, genetic components (mostly DNA and RNA), virulence factors (e.g., toxins, adhesive and invasive enzymes), metabolites and signalling compounds such as QS molecules (264, 275-278). Among the OM derived proteins, Kesty *et al*. have detected a number of proteins incorporated abundantly in OMVs such as OmpA, OmpC, and OmpF (279). Consistently, alkaline phosphatase and AcrA (a protein component of efflux pump), which are examples of periplasmic proteins, have also been detected in OMVs (274). Interestingly, the OMVs and bacterial OM are highly similar in term of their lipid profiles, however little known about the types and the ratio of OMVs lipid into that of OM (280). Therefore, one of the aims of this Ph.D. study is to evaluate the lipid composition of OMVs in the presence and absence of the membrane stressor (polymyxin B).

1.4.1 Mechanisms of OMVs biogenesis:

Although the OMVs have been studied extensively, however the precise mechanism (s) of OMVs production by Gram-negative bacteria have not been fully understood (280). To date, four rational models for biogenesis of OMVs are proposed (**Figures 1.11 and 1.12**). The first model suggests that OMVs are initiated when a localised expansion of OM occurs faster than that of the underlying peptidoglycan layer which in turn results in losing the integrity of the envelope and separation of the peptidoglycan due to a weakness in lipoprotein cross-linkages (281, 282). This model is supported by evidence that the lipoprotein contents of *E. coli* MOVs are significantly lower than that of corresponding bacterial cell which indicates that the OMVs are generated at the weak sites of OM-peptidoglycan linkage such as cell division regions (281). Another supporting evidence showed that the lipoprotein contents of *P. aeruginosa* OM are less than that of *E. coli*, thus the OMVs production by *P. aeruginosa* cells is usually higher than that produced by *E. coli* (283, 284). Further supporting observations demonstrated that mutations in genes encoding Tol-Pal system (a system responsible for OM-peptidoglycan cross-linking and OM integrity) of Gram-negative bacteria increases OM vesiculation (285, 286). In addition, Song *et al*. found that *V. cholera* overproduces vrrA gene, which is a small non-coding RNA gene, in response to stressful environmental conditions. Overexpression of these vrrA genes increases the production of OMVs via reduction of the OmpA (287). The second MOVs generation model is linked to the periplasmic accumulation of peptidoglycan fragments (272). During the turnover of peptidoglycan, fragments of low molecular weight peptidoglycan are generally accumulated in the cytoplasmic space prior to their cytoplasmic translocation by permease enzyme; and some of these fragments have been observed in secreted vesicles (272, 288, 289). This peptidoglycan accumulation applies a turgor pressure on *Porphyromonas gingivalis* OM resulting in bulging of the membrane and releasing of OMVs (272). To support this model, Zhou *et al*. examined the released OMVs from *P. gingivalis* and observed that these OMVs contain a muramic acid which is one of the peptidoglycan constituents (272). Another study had been conducted by Hayashi *et al*. to support this model, they found that mutation of

peptidoglycan hydrolase could lead to the accumulation of peptidoglycan intermediates within the periplasmic space which inhibit the degradation of the cell wall components of *P. gingivalis* and increase the production of OMVs (290). Despite the supporting pieces of evidence, this model remains a subject of debate because no study yet performed to correlate the exerted turgor pressure on the OM with the level of peptidoglycan inside the OMVs of *P. gingivalis*; and whether the peptidoglycan fragments are present in other strains or they are a species-specific fragments (284). The third model proposed the presence of molecules, namely B-band LPS and the PQS (*Pseudomonas* quinolone signal) molecules, that induce the curvature of the OM (291, 292). Some earlier studies showed that the presence of truncated LPS in OM of *P. aeruginosa* and *Salmonella* induces blebbing of the membrane (293, 294). Subsequently, further studies have been conducted based on these observations to confirm that the electronegativity of B-band LPS in *P. aeruginosa* MO induces electrostatic repulsion, which provokes membrane blebbing and ultimately OMVs formation (291). Molecular analysis of different strains of *P. aeruginosa* demonstrated that they carry different phenotypes of LPS (e.g., A^+B^+, A^-B^+, A^+B^- and A^-B^-); and the hypervesiculated strains display the negatively charged A-B-band LPS (295). Therefore, the enrichment of OMVs with negatively charged B-band LPS rather than positively or neutrally charged LPS provides a supporting evidence for this model (291, 295-297). In addition, it has been shown that PQS molecules play an important role in blebbing of *P. aeruginosa* OM and release of OMVs (284). These molecules destabilize Mg^{2+}/Ca^{2+} salt bridges of the LPS via sequestering the positive charges of the divalent cations and induce a repulsion of LPS molecules, which results in membrane blebbing and vesiculation (298). The addition of external Mg^{2+} to *P. aeruginosa* culture increases the production of OMVs, which gives further supporting evidence for the role of PQS molecules in enhancing OMVs biogenesis (284). This third model had been proposed by Kuehn *et al*. based on all of these observations (299). However, this model is a species-specific because it was conducted mostly on *P. aeruginosa*; and some other strains such as O-antigen mutant strains of *P. gingivalis* lack B-band LPS (300, 301). Although, in all of these three proposed models, OMVs are the products of envelope

instability, however a contradictory observation has been seen with *E. coli* OMVs production. A genetic study performed on *E. coli* by McBroom *et al*. revealed that OMVs biogenesis is uncorrelated to membrane instability (302), therefore OMVs production could be a combination of different mechanisms rather than single one.

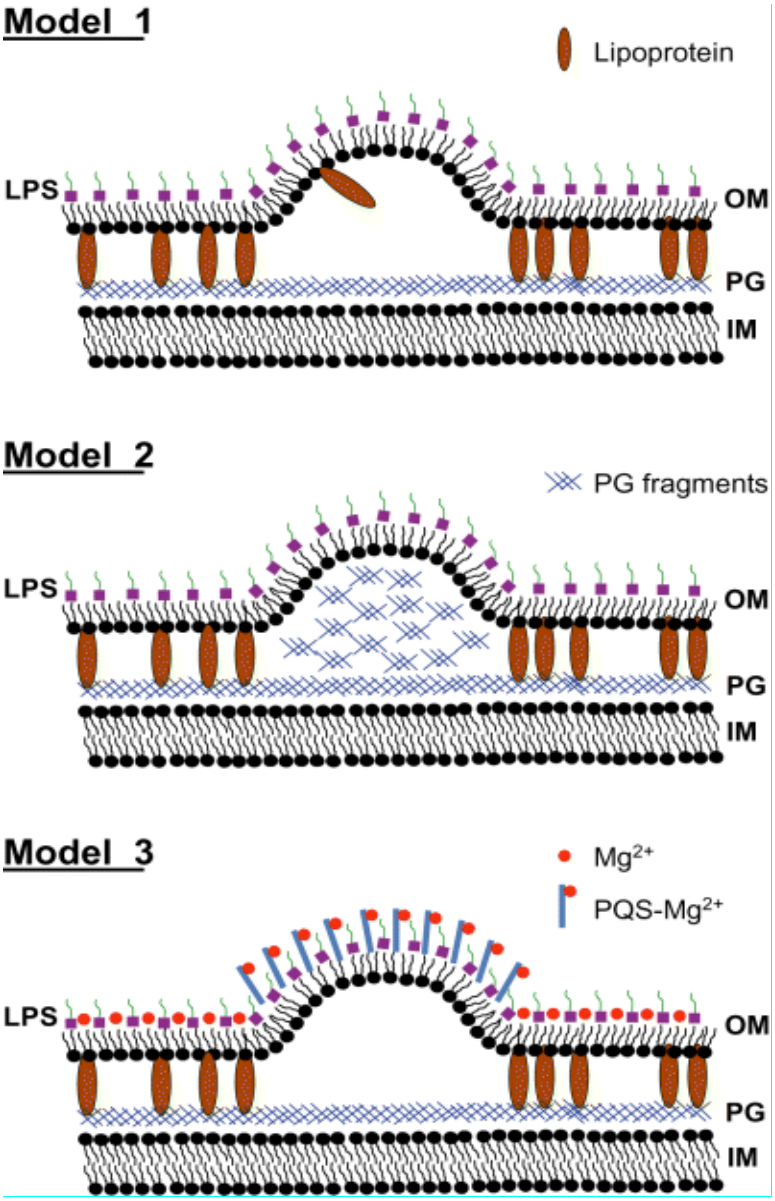

Figure 1.11 Suggested models for OMVs biogenesis. First model: OMVs production is initiated at OM specific regions where the OM expands quicker than underlying peptidoglycan layer as a result of lipoprotein lacking. Second model: inefficient cytoplasmic translocation of peptidoglycan fragments results in accumulation of these fragments in the periplasmic space and subsequently increasing OM blebbing. Third model: PQS-$Mg2^{+}$ interaction in *P. aeruginosa* induces charge-to-charge repulsion and OM blebbing. PG, peptidoglycan; IM, inner membrane. (Figure adapted from Mashburn-Warren *et a*l. (284) with permission).

More recently, Roier *et al*. have proposed a novel general model for the biogenesis of OMVs (303). In this model, OMVs production depends on the accumulation of phospholipid in bacterial OM which requires the involvement of the phospholipid transport system (ATP-binding VacJ/Yrb ABC transport system). Mutation of VacJ/Yrb gene induces overproduction of OMVs in *Haemophilus influenzae* and *V. cholera* (303). The authors also found that this mechanism of OMVs biogenesis is affected by iron availability, where low iron level downregulate OMVs level. This phospholipid-associated model could be a general model for all Gram-negative bacteria and acts in conformity with previously mentioned models. In addition, it overcomes the species-specific limitation associated with those models (303).

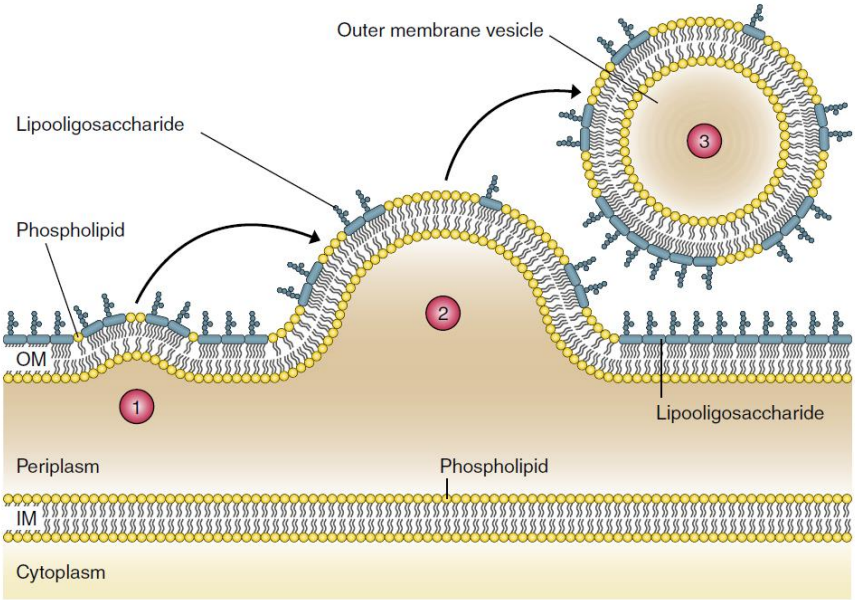

Figure 1.12 Phospholipid-based general model for biogenesis of Gram-negative bacterial OMVs. First step: phospholipid accumulated in the outer layer of MO as a result of suppression of vacJ and/or yrb genes which induces outward membrane blebbing. Second step: membrane blebbing is further induced by LPS-associated ionic interaction. Third step: enrichment of the liberated OMVs with phospholipids. (Figure adapted from Roier *et al*. (303) with permission).

1.4.2 Principal components of OMVs:

Structural and chemical analysis of OMVs cargo showed that they contain mainly proteins and lipids; in addition to some nucleic acids, metabolites and QS molecules (273). OMVs proteomic

analysis has been performed extensively in different Gram-negative bacterial strains and found that there is some difference between OMVs proteins and proteins of other cellular parts (282). Mass-spectrometric analysis of OMVs proteome has revealed that OMVs proteins come predominantly from OM and periplasmic proteins (304, 305). In addition, several other studies conducted on different strains showed that some proteins in OMVs are cytoplasmic and cytoplasmic membrane proteins (264, 278, 306). Functionally, OMVs proteins have been sorted into multiple functional classes including OM structural protein, transporters, porins, enzymes and stress response proteins (278, 280). OMVs are considered as an important extracellular reservoir for many enzymes (e.g. peptidases, nucleases, proteases and β-lactamases) (278, 307-309). Although, it has been found that sorting of the proteins into OMVs differs by the difference in growth phases at which the OMVs are harvested, and by the presence or absence of stressors, however, the mechanism of protein sorting is yet unclear (310). Similarly, lipidomic studies showed that the main lipid component of OMVs are LPS and phospholipids (273). The anionic B-band of LPS is the predominant component of the OMVs outer leaflet but not the neutral A-band or the whole LPS molecule (291, 311). By MS analytical study, Haurat *et al*. have found that lipid A was sorted in OMVs from *P. gingivalis* in its deacetylated form which is different from that of the parent cell (300), however, both OM and OMVs lipid profile were similar in a related species (*Bacteroides fragilis*) (312). Moreover, the OMVs membrane also contains phospholipids and they differ from that of the OM of the parent bacteria (311, 313). In *P. aeruginosa*, the abundant phospholipid in OM is phosphatidylethanolamine whereas in OMVs is phosphatidylglycerol (314). Furthermore, saturated fatty acyl chains were also found in relatively higher amount in OMVs compared to OM which increase the rigidity of OMVs (314, 315). In addition to the proteins and lipids, the cargo of OMVs also contains many other bioactive molecules such as DNA (circular plasmid DNA, linear plasmid DNA, phage DNA and chromosomal DNA), RNA, ions, signalling molecules, peptidoglycan muramic acid and other metabolites (269, 275, 291, 316, 317).

1.4.3 Functions of OMVs:

OMVs act as a decoy for many toxins, adhesins, and immunomodulatory substances, therefore they play an essential role in the pathophysiology of bacteria **(Figure 1.13)** (279, 291, 318-321). An example of toxins that have been demonstrated in OMVs are Heat labile enterotoxin from enterotoxigenic *E. coli*, VacA from *Helicobacter pylori*, Shiga toxin from *Shigella dysenteriae*, ClyA from enterohemorrhagic *E. coli* and hemolysin from *Salmonella typhi* (274, 279, 319-323). Surprisingly, some of these OMVs-incorporated toxins become more advantageous than their cellular counterparts (273), Wai *et al*. have demonstrated that the periplasmic inactive ClyA monomer becomes active ClyA oligomer within the OMVs which is essential for polymerisation of the monomer (320). In addition, OMVs-packed adhesins facilitate the adhesiveness of OMVs to the host cell membrane and delivery of OMVs contents into the mammalian cell either by fusion or by uptake mechanism (279, 324, 325). Simultaneously, OMVs increase the lifespan of their cargo by protecting them from host destroying enzymes and antibodies (326). Several studies have reported that OMVs modulate host immune system by delivering various immunomodulatory proteins and lipids (327-329). Moreover, it has been suggested that OMVs augment bacterial virulence by enhancing biofilm formation and co-aggregation of bacterial cells, but the exact mechanism of this actions need to be investigated (273, 330, 331).

Interestingly, OMVs play a crucial role in bacterial responses to chemical and physical stressors and nutritional starvation (273). As an attempt to overcome the stressful effects of temperature, antibiotics, bacteriophages or antibodies, bacteria try to increase the production of OMVs suggesting their role in titrating these stressors (264, 302, 328). OMVs protect bacteria from the action of antibiotics either by their cargo of antibiotic-degrading enzymes such as β-lactamase and gentamycin-inactivating enzymes or by sequestering the antibiotics such as polymyxins and other membrane-active antibiotics (190, 291, 313, 332). Furthermore, a proteomic study conducted by Park *et al*. reported that *P.s aeruginosa* growing in biofilms secretes OMVs enriched with drug-targeting proteins, such as efflux pump proteins, compared to OMVs

isolated from planktonic *P. aeruginosa*. Such finding provides another explanation for the role of these vesicles in drug resistance (333). Besides that, bacteria produce OMVs concentrated with essential metal ions, complex biomolecules-degrading enzymes and ATP synthesis machinery which make OMVs rich source for bacterial nutrition during the starvation (264, 278). In another function, OMVs act as a weapon to kill another bacteria, for example Gram-negative bacteria secretes OMVs to kill Gram-positive in mixed communities (334). Various studies have demonstrated that OMVs carry bacteriolytic enzymes such as peptidoglycan hydrolases and endopeptidase which capable of distinguishing and targeting the non-self-cells in polymicrobial environments (157, 335). Also Kadurugamuwa *et al*. have observed that OMVs could be used as antibiotic reservoirs to deliver the drug in high concentration at the site of infection which might be a promising approach to reduce the antibiotic resistance and assure the access of antibiotic to the target site (291). Very recently, Kim *et al.* have reported that OMVs could potentially destroy the fully established tumour cell via induction of long-term immune response. This phenomenon depends on the OMVs-induced production of cytokines and interferon-γ that capable of killing cancer cells without detectable harmful effects (336).

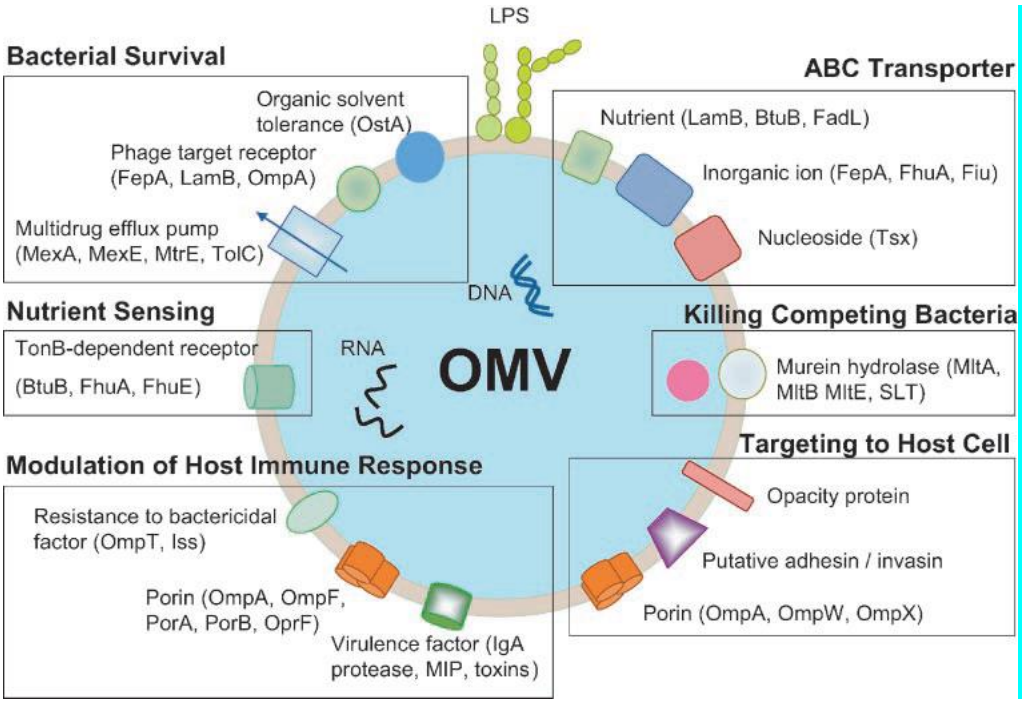

Figure 1.13 Proposed pathophysiological functions of OMVs isolated from Gram-negative bacteria. The predicted functions were obtained from the proteomic analysis of pathogenic and non-pathogenic bacterial OMVs. (Figure adapted from Lee *et al*. (280) with permission).

1.5 Proteomics: an overview

Although the first simple proteomic analysis and protein mapping started in 1975 using two-dimensional-polyacrylamide gel electrophoresis (2DPAGE) (337-339). However, the term "proteomics" have been introduced to the molecular biology for the first time in 1995 to characterise the complete cellular proteins in global-scale techniques (340). In early proteomic studies, the 2DPAGE was utilised extensively to identify bacterial proteins, but due to lack of the sensitivity of protein sequencing and time consumption, this technology never satisfied the proteomic analysis of complex biological samples even with the introducing of micro-sequencing technologies (341). By the development of mass spectrometry (MS) approach, the proteomic field entered a new era (342). Fortunately during the 1990s, and with the emergence of complete genomic sequencing, the high-sensitivity high-throughput MS-based facility became highly applicable in proteomic studies (343). Interestingly, the central dogma of MS-based proteomic procedure is the liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). In this platform, the enzymatic digestion of complex proteins and

production of peptides occurs prior to chromatographic separation and MS identifications (344). Nowadays, thousands of proteins can be measured within a short time using the futuristic proteomic techniques (345). As a part of metabolomics, the proteomics should be connected and communicated with other omic studies such as genomics, which focuses on genetic mapping and gene array; and lipidomics, which investigates the structural and functional changes of lipids. These associations of omic studies provide a complete set of information about changes in metabolic pathways that associated with pathophysiological conditions **(Figure 1.14)** (346).

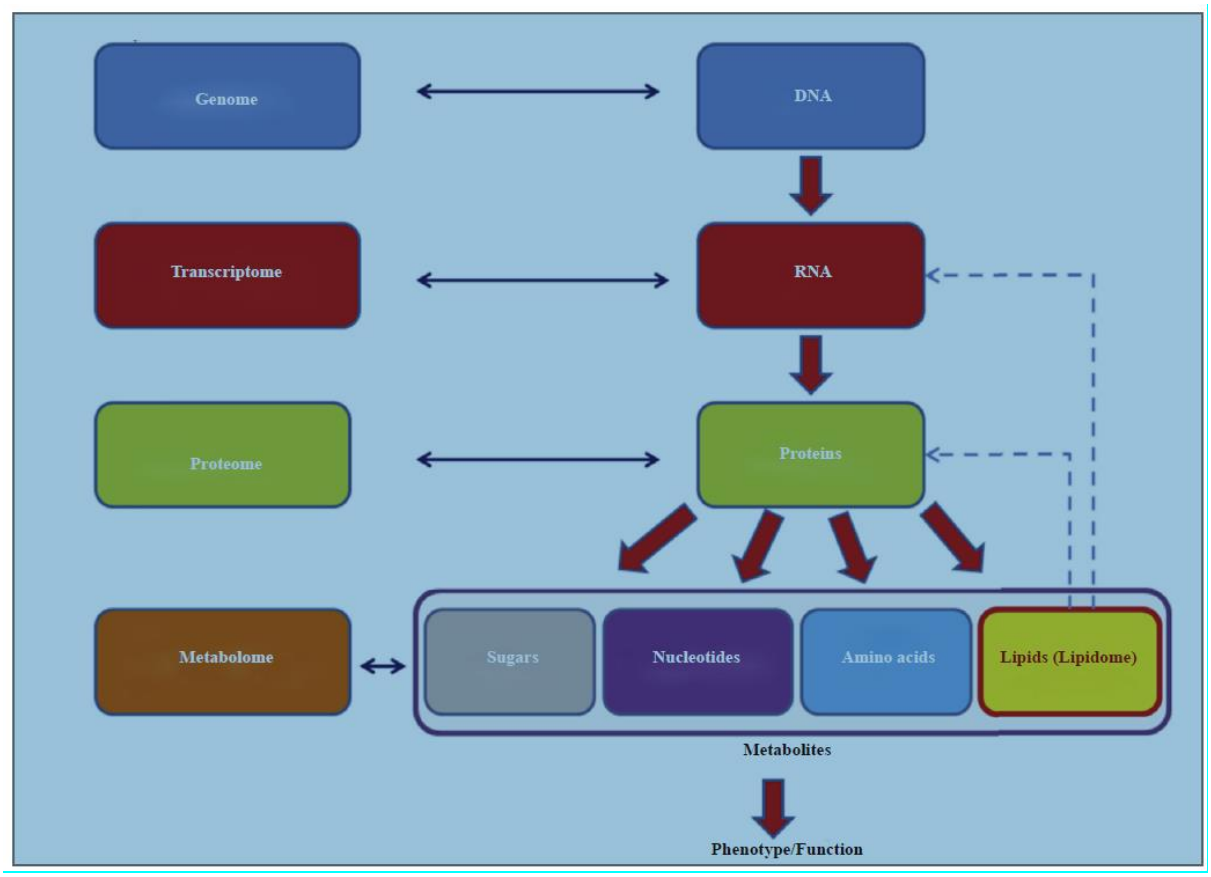

Figure 1.14 Schematic presentation of the communication of the lipidome with genome, proteome, transcriptome and metabolome. Lipids can regulate protein function and gene transcription as part of a dynamic interaction (Figure adapted from Koriem *et al*. (347) with permission).

1.5.1 Types of Proteomics

Currently, several analytical studies are grouped under the rubric of proteomics **(Figure 1.13)**.

1.5.1.1 Protein expression proteomics

Expression proteomics is usually employed to quantitatively analyse protein expression mainly when there is a variability in protein samples. The comparison of protein expression for the total or part of proteome can be performed by using such technique, and the information achieved from this proteomics studies can be utilised in the identification of disease-associated proteins or discovery of novel signalling proteins (348).

1.5.1.2 Structural proteomics

This type of proteomics aims to physically identify the cell map through mapping out the structure of proteins in protein complexes or proteins within particular subcellular organelles (349). Besides, these proteomic studies endeavour to characterise the entire proteomes within the complex protein samples, identify the subcellular localisation of the proteins, and determine proteins interactions. Collectively, the data obtained from structural proteomics aid in revealing the role of proteins in cell architecture and unique characterisation (348).

1.5.1.3 Functional proteomics

This proteomics is a general term covers many proteomics technologies such as affinity chromatography for isolation of distinct proteomes or sub-proteomes for further studies. “Functional proteomics” give a high opportunity for better understanding of protein signalling, mechanism of diseases and protein-drug interactions (348).

1.5.2 Biological applications of proteomics

Proteomics is highly applicable in all biological systems **(Figure 1.14)**.

1.5.2.1 Identification of Protein Complexes

Identification of proteins is considered as one of the major applications of proteomics in the biological field and provides detailed information about protein complexes, which can be

utilised in further investigations. Isolation and identification of proteins by proteomic studies depend on protein-specific criteria. Importantly, the identified proteins give an idea about the results of subsequent analysis such as the validity of the applied biochemical methods (348).

1.5.2.2 Protein Expression Profiling

Proteomics is broadly applied to study the expression profiling of proteins. The determination of signalling proteins or disease-specific proteins can be achieved merely by expression profiling via proteomics approach. Expression profiling is usually performed by using two-dimensional electrophoresis (2-DE), isotope-coded affinity tags (ICAT), or protein array (348). Protein expression profiling of bacteria could provide a comprehensive idea about actual cellular responses to a wide variety of stressors including antibiotics from different classes, and push forward the antibiotic discovery process (350).

1.5.2.3 Protein Phosphorylation

Protein phosphorylation is a common Posttranslational modification process occurs virtually in almost all biological systems. The identification and characterisation of phosphorylated protein is paramount for better comprehension of protein function (351, 352). MS-based proteomics is a useful approach for identification of not only phosphoproteins but also all other types of protein modification. Identification of novel phosphorylated proteins, measurement of effector-associated changes in the state of phosphorylation, determination of the site of protein phosphorylation, and all other phosphorylation changes can be studied simultaneously by using MS-based phosphoproteomics. Moreover, knowledge about enzymes (e.g. kinases and phosphatases) and enzymatic regulations can be achieved via determination of phosphorylation sites of protein (348).

1.5.2.4 Proteome Mining

Proteome mining is a kind of functional proteomics technology applied to obtain information about the protein from the sub-proteomes analysis. The core idea of this technique is the

selective competition between drug-like molecules and naturally occurring cellular ligand for a specific target site on the protein (348). In a study conducted on MDR *Listeria monocytogenes* strain, proteomics mining had been utilised to identify 168 drug-targeted proteins; one of these proteins was directly involved in peptidoglycan biosynthesis pathway and known as penicillin-binding protein 4. In the same study, four of particular interest compounds have been investigated and found to be as promising multi-targeting agents (353). Similarly, Lohani *et al.* have used proteome mining to determine three multi-enzymatic proteins in *Clostridium difficile*, these proteins could be a good target for new antibiotics against this serious pathogen (354). In addition, several proteomics studies have been performed to investigate the importance of proteomics approaches in determining the expressed proteins that are responsible for antibacterial drug resistance (355, 356). In one of these studies, proteomic analysis of colistin-resistant and -susceptible *A. baumannii* strains revealed that colistin treatment of colistin-resistant *A. baumannii* strains expressed many proteins that enhanced their survival against colistin compared to susceptible strains (357). Collectively, proteomics techniques are of great importance in all biological systems. In this PhD study, protein expression proteomics has been utilised to identify the compositional differences of the OM and OMVs sub-proteome between polymyxin-susceptible and -resistant *K. pneumoniae* isolates, and to investigate the effect of polymyxin B treatment on OMV sub-proteome that are selectively packaged from the parent bacteria into the OMVs.

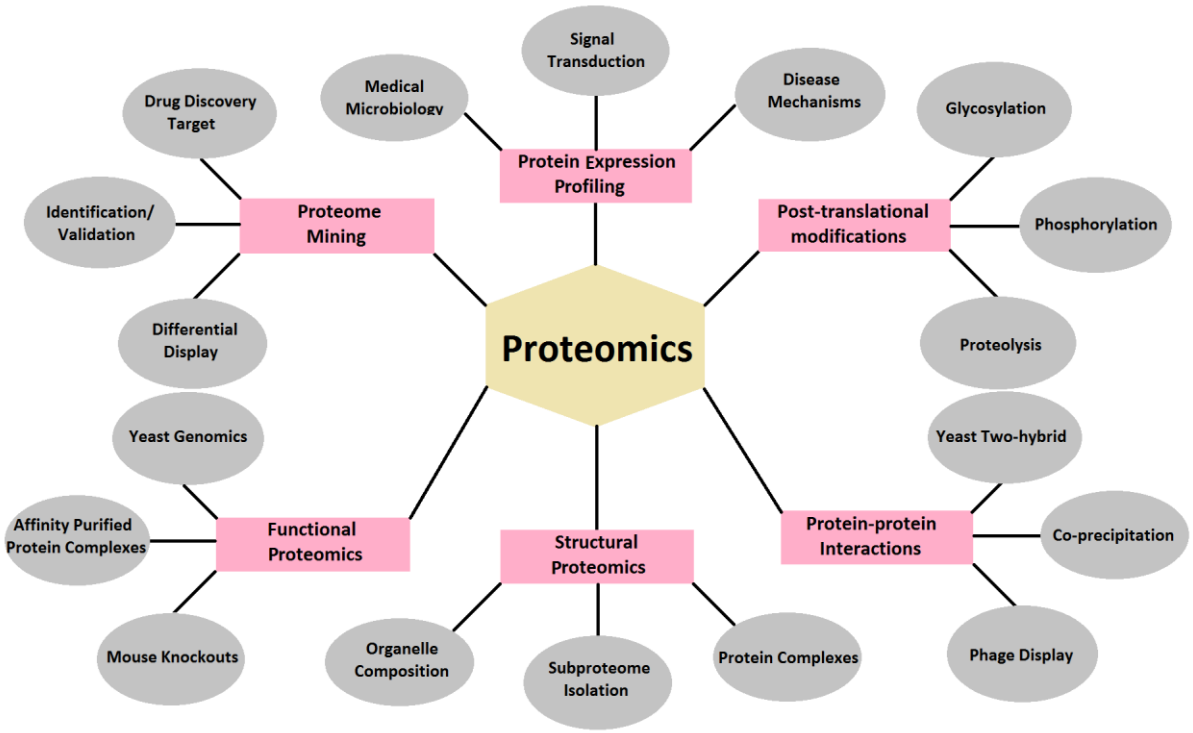

Figure 1.15. Different types of proteomic and their biological applications. (Figure adapted and modified from Graves *et al*. (348) with permission).

1.6 Lipidomics: an overview

Lipidomics is recently introduced by Han *et al*. in 2003 as a branch of metabolomics that covers the complete qualitative and quantitative characterisation of lipid molecules (lipidome) from different biological systems including serum, plasma, cell or the whole organism (358, 359). In another word, lipidomics is a large-scale-based structural and functional analysis of lipids and their association with other cellular components such as proteins and other metabolites that are connected with lipid metabolic pathways and functions (360). The lipidomics-based identification of changes in lipid metabolism is crucial in determining the underlying biochemical mechanism of lipid-associated diseases (347). With the development of lipidomics, lipid analytical studies are directed from single lipid molecule characterisation toward global characterisation of all lipid metabolites within the biological samples to gain a clear insight about the pathophysiological role of lipids (358). Recently, many organisations and online lipid databases have been initiated to encourage researchers in this field. These resources encompass a wealth of information on structures, functions and classes of lipids. For instance, “Lipidomics

Expertise Platform" which was launched in 2005 (http://www.lipidomics-expertise.de), European Federation for the Science and Technology of Lipids (http://www.eurofedlipid.org), Lipidomics Research Center Graz (http://omicscentergraz.at), LIPID MAPS (http://www.lipidmaps.org), LIPID BANK (http://lipidbank.jp), Lipid Data Bank (http://www.caffreylabs.ul.ie), Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/), Cyberlipid Center (http://www.cyberlipid.org) and the Lipid Library (http://www.lipidlibrary.co.uk) (361).

1.6.1 Analytical approaches for lipidomics and data processing

Traditionally, high-performance liquid chromatography (HPLC) and gas chromatography (GC) were used for analysis of lipid samples (362-364). Usually, prior to the separation of lipid classes into single molecules by HPLC or GC, the lipid samples are fractionated into classes by thin-layer chromatography (TLC), normal-phase liquid chromatography (NPLC) or solid-phase extraction (SPE) (365-367). Although, different classes of lipids can be studies with such "classical" lipidomic approaches. However, they are associated with a number of limitations such as the dearth of sensitivity, limited resolution (only a particular number of individual molecular species can be analysed); and time consumption (especially with GC as many lipids are gas-immiscible and need to be converted to miscible compounds) (361). These limitations have been largely addressed by the emergence of advanced lipidomic technologies such as electrospray ionization (ESI), matrix-assisted laser desorption/ionization (MALDI) and atmospheric pressure chemical ionization (APCI). Therefore, the analysis of thousands of lipids becomes possible in one technique with high resolution and sensitivity (361). Recently, these technologies have been developed further and connected to MS. For example, direct-infusion ESI–MS and ESI–MS/MS, and MALDI combined with Fourier transform ion cyclotron resonance MS (MALDI–FTICR–MS) or time-of-flight–MS (MALDI–TOF–MS). More recently, nuclear magnetic resonance (NMR) spectroscopy has also been used in the lipidomic field (361, 368). Even with the advanced lipidomic technologies, identification of all lipids

within a sample by using single technique is a challenge due to the huge diversity of lipid properties including structures, concentrations and physicochemical natures. Hence, multiple techniques applied simultaneously in lipids identification studies (361).

Interestingly, with the rapid development of lipidomic analytical approaches, an abundant amount of raw data can be collected within a short period particularly with untargeted lipidomics. Thus, suitable computerised bioinformatics methods are required to analyse these raw data (chromatographic and mass spectral data) and to identify and quantify lipids (369). The first step in processing MS-based lipidomics data is the identification of lipids via a software. Many software packages have been established during the last ten years such as Lipid Inspector, Lipid View, LipidXplorer, *m/z* Mine 2, and Lipid Data Analyzer (370). Additionally, identification and quantification of lipids can also be achieved by IDEOM (metabolomics data processing software) (371). Secondly, a set of internal standards is utilised to normalise the data, then quantification of identified lipid is accomplished by comparing these lipids with the internal standards (372). In the third step, sets of the data obtained from the second step are then analysed statistically. In lipidomic statistical data analysis, group comparisons are usually performed using Univariate statistical methods in particular Principal component analysis (PCA) (373). With the development of analytical and statistical software, more information about structural and functional characteristics of lipids will be available. Consequently, the mechanisms of lipidome-associated disease will become approachable (374).

1.6.2 Types of lipidomics

Based on MS spectra, lipidomic analysis can be divided into ‘‘untargeted’’, ‘‘focused’’ and ‘‘targeted’’ lipidomics **(Figure 1.16)** (375). In untargeted lipidomics, all lipids in the extracted samples can be detected without prior information about their ions or fragmentations. This approach depends on grouping the molecular ions by subjecting the detected peaks to additional software analysis (376). The most applicable techniques for untargeted lipidomics that provide a high resolution and accuracy, are High-resolution Fourier-transform MS (FT-MS), quadrupole

ion trap (Q-IT) and quadrupole-time-offlight (Q-TOF) (377). On the other hand, in focused lipidomics, the analysis of lipid samples depends on focusing on molecules within a selected categories using specific fragments which are either product ion scanning (for detecting fragments of required ions), precursor ion scanning (applied for detection of lipids within same class or subclass) or neutral-loss scanning (for detecting lipids that losing same fragment). This method is highly sensitive due to the reduced basal noise. Thus, the significant minor molecules can be detected by focused lipidomics technique (368). The third strategy is the targeted lipidomics which is used to analyse specific molecules via utilising information about both the targeted fragments and their parent ions; also this method requires the application of MS2 spectrometer. In this strategy, the mass spectrometer is operated in either selected reaction monitoring (SRM) or multiple reaction monitoring (MRM), and it is mostly applied to identify lipids that have known fragmentation patterns. Targeted lipidomics technology is commonly applied for studying drugs of abuse and signalling lipids (375). In all the three lipidomics technologies, the lipid analysis can be accomplished with or without chromatographic-mass spectrometer pairing. Although, the untargeted lipidomics is less sensitive than the other two techniques due to the high baseline noise. Nevertheless, it results in the identification of a considerable number of compounds in a single run and provides a high opportunity for identifying unanticipated lipid molecules. Whereas, the targeted lipidomics is associated with remarkable sensitivity (375).

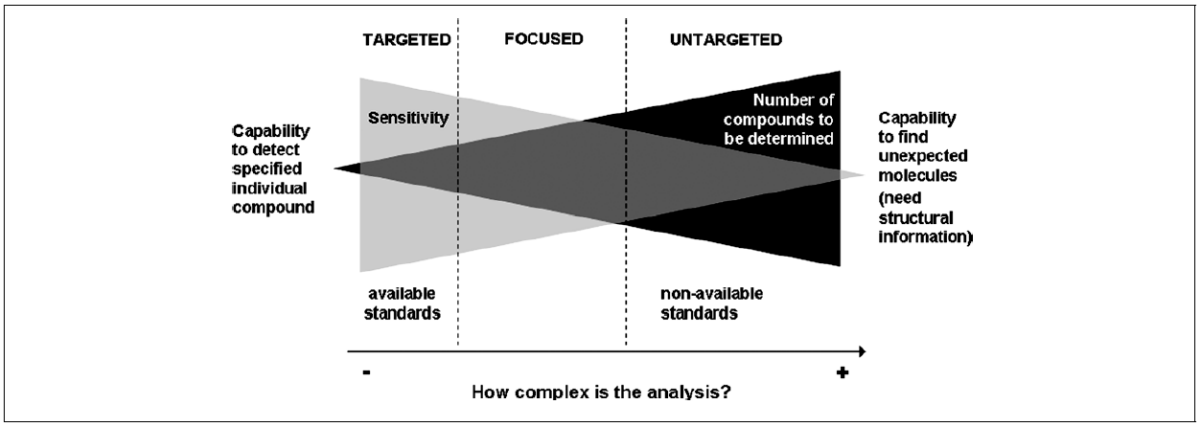

Figure 1.16 Different lipidomics technologies and their capability to detect the individual molecular species. (Figure adapted from Navas-Iglesias *et al*. (375) with permission).

1.7 Summary

The worldwide use of antibiotics results in the development of bacterial resistance, which poses a threat to the healthcare. With the dearth in the discovery and development of new antibiotics and rapid emergence of MDR pathogens (particularly MDR Gram-negative bacteria) in clinical situations, the optimisation of clinically available antibiotics is highly warranted. For this critical situation, polymyxins, an "old-antibiotic" have been re-birthed as the last-resort therapeutic option for the treatment of MDR Gram-negative bacterial infections. Unfortunately, many PK/PD studies of polymyxins demonstrate that the administration of polymyxins as a single therapy is unlikely to achieve optimal and safe plasma concentration particularly with highly resistant pathogens. To overcome these limitations with polymyxins therapy, a combination with other antibiotics is an option. Despite of different models have been suggested for the mechanism of polymyxin action, however the precise mechanism remains unclear. Better understanding the mode of actions of polymyxin combination therapy is crucially important to achieve maximum benefit. In this PhD study, combination therapy, proteomics and lipidomics strategies were applied to provide further details about the mechanisms of action and resistance of polymyxin against MDR pathogens.

1.8 Hypotheses and aims

The central **hypotheses** to be tested in this research were that:

1. That the superior polymyxin combination synergistically kills MDR Gram-negative pathogens, whereby the second antibiotic enhances bacterial killing of polymyxins and minimises the emergence of polymyxin resistance (objective 1).
2. That polymyxin treatment significantly affects the protein profile of OMVs isolated from both polymyxin-susceptible and -resistant XDR *K. pneumoniae* isolates (objective 2).
3. That polymyxin treatment significantly affects the lipid cargo of OMVs derived from both polymyxin-susceptible and -resistant MDR *K. pneumoniae* isolates (objective 3).

4. That polymyxin treatment significantly affects the protein profile of OM isolated from both polymyxin-susceptible and -resistant XDR *K. pneumoniae* isolates (objective 4).

The **aims** of this thesis were:

- Identify a superior polymyxin combination against MDR Gram-negative pathogens that are the common cause of CF lung infections using broth microdilution checkerboard method and static time-kill studies (Chapters 2). The MICs of polymyxin B and silver NPs against MDR Gram-negative isolates were tested. polymyxin B combination with silver NPs was investigated via in vitro synergy testing methods including broth microdilution checkerboard method and static-time kill studies.
- To perform a comparative analysis of the lipidome of OMVs isolated from both polymyxin-susceptible and -resistant *K. pneumoniae* isolates and to identify key lipid molecular species that are selectively packaged from the OM into the OMV sub-lipidome of the resistant isolates.
- To perform a comparative analysis of the proteome of both OM and OMVs isolated from both polymyxin-susceptible and -resistant *K. pneumoniae* isolates and to identify key proteins that are selectively incorporated into OM and OMV from the resistant isolates.

1.9 Significance statement

- The combination of polymyxin B and silver NPs excretes significant synergistic activity against MDR Gram-negative isolates particularly polymyxin-resistant *P. aeruginosa* isolates that is commonly colonise CF lung.
- Silver NPs compromise the integrity of the OM through disruption of disulfide bonds within inner membrane respiratory enzymes, therefore, the combining silver NPs with polymyxin B could potentially enhance the bactericidal effect of polymyxin B via disruption of MO permeability.

- Polymyxin B significantly affects the lipid profile of OMVs isolated from both polymyxin-susceptible and -resistant *K. pneumoniae* isolates with much more effect observed on lipid cargo of resistant isolates which might suggest that polymyxin B could potentially alter the virulence of resistant strains via alteration of OMVs lipid profile.
- The protein contents of both OM and OMVs isolated from polymyxin-resistant *K. pneumoniae* isolates significantly altered by polymyxin B treatment.

Overall, the findings from this PhD study significantly provide highlighted insights on the synergistic mechanism of polymyxin combination. In addition, the significant information provided in this study greatly facilitate the better understanding of the mechanism of drug-pathogen interactions.

1.10 Structure of the thesis

Chapter 1 is the background of this thesis. Chapter 2 of this thesis focus on the study of synergistic combination of polymyxin B and silver NPs, while chapter 3, 4 and 5 focus on the study of the effect of polymyxin B on sub-proteome and lipidome of OMVs and OM of *K. pneumoniae*. The works from chapter 2 and 4 comprise of manuscripts that have already been published. The works from chapter 3 and 5 comprise of manuscripts that have already submitted for publication. The text and figures in these chapters have been reproduced as published or submitted for publication with modifications for this thesis as a requirement for the research degree. The final chapter provides a conclusion and future directions.

Chapter 2: A fresh shine on cystic fibrosis inhalation therapy: antimicrobial synergy of polymyxin B in combination with silver nanoparticles

Article

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A Fresh Shine on Cystic Fibrosis Inhalation Therapy: Antimicrobial Synergy of Polymyxin B in Combination with Silver Nanoparticles

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This *in vitro* study aimed to investigate the synergistic antibacterial activity of polymyxin B in combination with 2 nm silver nanoparticles (NPs) against Gram-negative pathogens commonly isolated from the cystic fibrosis (CF) lung. The *in vitro* synergistic activity of polymyxin B with silver NPs was assessed using the checkerboard assay against polymyxin-susceptible and polymyxin-resistant *Pseudomonas aeruginosa* isolates from the lungs of CF patients. The combination was also examined against the Gram-negative species *Haemophilus influenzae*, *Burkholderia cepacia*, *Burkholderia pseudomallei*, *Stenotrophomonas maltophilia*, *Klebsiella pneumoniae* and *Acinetobacter baumannii* that are less common in the CF lung. The killing kinetics of the polymyxin B-silver NPs combinations was assessed against *P. aeruginosa* by static time-kill assays over 24 h. Polymyxin B and silver NPs alone were not active against polymyxin-resistant (MIC $\geq$ 4 mg/L) *P. aeruginosa*. Whereas, the combination of a clinically-relevant concentration of polymyxin B (2 mg/L) with silver NPs (4 mg/L) successfully inhibited the growth of polymyxin-resistant *P. aeruginosa* isolates from CF patients as demonstrated by $\geq$ 2 $\log_{10}$ decrease in bacterial count (CFU/mL) after 24 h. Treatment of *P. aeruginosa* cells with the combination induced cytosolic GFP release and an increase of cellular reactive oxygen species. In the nitrocefin assay, the combination displayed a membrane permeabilizing activity superior to each of the drugs alone. The combination of polymyxin B and silver NPs displays excellent synergistic activity against highly polymyxin-resistant *P. aeruginosa* and is potentially of considerable clinical utility for the treatment of problematic CF lung infections.

KEYWORDS: *Silver, Nanoparticles, Cystic Fibrosis, Antibiotic Combination, Polymyxin.*

2.1 Introduction

Medicine is entering a critical period where bacteria have developed resistance to almost all clinically available antibiotics and at the same time the antibiotic pipeline continues to dry up (378). This series of unfortunate events has been dubbed 'the perfect storm'(379). CF patients whom commonly suffer from with chronic polymicrobial lung infections are dependent on antibiotics on a daily basis; and are one of the groups at highest risk from MDR bacterial infections. CF is caused by dysfunction of the trans-membrane conductance regulator (CFTR) protein resulting in impaired chloride transport (380). The defective CFTR function leads to the accumulation of a thick mucus in the lungs which promotes bacterial infections, most commonly *Pseudomonas aeruginosa*, found in ~80% of CF patients (381). *Haemophilus influenzae*, *Stenotrophomonas maltophilia, Burkholderia cepacia, B. pseudomallei, Klebsiella spp*. and *Acinetobacter baumannii* less commonly infect the lungs of CF patients (381). *P. aeruginosa* is intrinsically refractory to antibiotics and as such infections are often problematic to treat (22). Once *P. aeruginosa* has established a chronic infection, it becomes increasingly resistant to the administered antibiotics; and up to 80–95% of CF patients die from respiratory failure, which is the culmination of chronic bronchiectasis caused by chronic airway infection and inflammation (381).

Silver and silver-containing compounds have well known antibacterial properties (382). Since the beginning of 19th century, silver has been used as an antimicrobial agent for the treatment of infectious diseases such as neonatal conjunctivitis (383). The antimicrobial application of silver gradually declined following the dawn of the antibiotics era (384, 385). However, due to the dramatic emergence of MDR bacterial 'superbugs,' the use of silver as an antimicrobial therapeutic has seen renewed interest (386). The use of ionic silver is limited as a result of its rapid inactivation and due to toxicity concerns (387). Silver NPs on the other hand hold great potential as antibacterial agents, due to their lower toxicity and good direct antibacterial activity against many problematic Gram-negative and Gram-positive pathogens (388, 389). Notably, silver NPs have been shown to display antimicrobial synergy when combined with β-lactam

antibiotics and membrane permeabilizing antimicrobial peptides such as gramicidin and magainin 2 (386, 390, 391) Recently, polymyxin B-capped silver NPs were shown to be of considerable utility as medical device coatings (392).

polymyxin B and colistin (polymyxin E) are lipopeptide antibiotics often used as last-line agents to treat infections caused by MDR Gram-negative pathogens (15). Colistin has been clinically used as the prodrug CMS for decades (146). Coly-Mycin® is a CMS nebulizer solution approved in Europe since 2003 for treatment of respiratory infections caused by *P. aeruginosa* in CF patients (393). Ominously, reports of polymyxin-resistant *P. aeruginosa* isolates from CF patient lung samples are becoming more and more common (394). Accordingly, it is important that novel strategies to prolong the efficacy of polymyxins are rapidly developed. The potential synergistic use of antibiotic combinations has emerged as a valuable means for prolonging the clinical efficacy of currently available antibiotics (238). The focus of the present in vitro study was to evaluate the antibacterial synergy and mode of action of silver NPs combined with polymyxin B against MDR Gram-negative pathogens that commonly infect the lungs of CF patients.

2.2 Methods

2.2.1 Materials

Polymyxin B (Catalogue number 81334, $\geqslant$ 6,500 IU/mg) was purchased from Sigma-Aldrich (Australia). Silver NPs, aqueous dispersion (2 nm, 2000 mg/L) were purchased from US Research Nanomaterials (TX, USA). All other reagents were purchased from Sigma-Aldrich (Australia) and are of the highest commercial grade available.

2.2.2 Bacterial isolates

MICs were determined by the broth microdilution method in accordance to the recommendations of the Clinical and Laboratory Standards Institute (395). Twenty-eight mucoid and nonmucoid clinical isolates of *P. aeruginosa* from patients with acute exacerbations

of CF **(Table 2.1)** (396). Twelve of the isolates were polymyxin-resistant and 16 are polymyxin sensitive. Five of the 28 isolates were resistant to at least three of the following agents: aztreonam, ceftazidime, meropenem, piperacillin, ticarcillin, gentamicin, tobramycin, and ciprofloxacin as we have previously characterised **(Table 2.2)** (396). A polymyxin heteroresistant reference strain, *P. aeruginosa* ATCC 27853 (polymyxin B MIC = 1 mg/L; American Type Culture Collection, Rockville, MD) was included as the reference strain. Resistance to polymyxin B was defined as MICs of ⩾8 mg/L (397). Six polymyxin-resistant (polymyxin B MIC > 128 mg/L) *Burkholderia* (*B. cepacia* and *B. pseudomallei*) isolates from CF patients were tested. A total of 11 *A. baumannii* isolates were examined in this study (6 polymyxin-susceptible strains and 5 polymyxinresistant strains), including a polymyxin-susceptible reference strain ATCC 19606 (polymyxin B MIC = 1 mg/L), polymyxin-resistant ATCC 19606R (polymyxin B MIC = 64 mg/L). Thirteen *K. pneumoniae* isolates were tested, including 11 polymyxin-resistant and 2 polymyxin-susceptible. The polymyxin-susceptible *K. pneumoniae* ATCC 13883 (polymyxin B MIC = 1 mg/L) was employed as the reference strain. For *S. maltophilia* 8 clinical isolates, including 7 polymyxin-susceptible and one polymyxin-resistant isolate FADDI-SM001 (polymyxin B MIC = 4 mg/L), were examined. Two polymyxin-resistant (polymyxin B MICs = 32 and 64 mg/L) *H. influenzae* clinical isolates were examined.

2.2.3 *In vitro* antibacterial synergy assessment

MICs were determined by the broth microdilution method in accordance to the recommendations of the Clinical and Laboratory Standards Institute (395). The time-killing kinetics of polymyxin B and in combination with silver NPs were tested against non-mucoid *P. aeruginosa* FADDI-PA066 (polymyxin B MIC = 64 mg/L), non-mucoid *P. aeruginosa* FADDI-PA067 (polymyxin B MIC = 16 mg/L), *K. pneumoniae* FADDI-KP027 (polymyxin B MIC = 128 mg/L) and *A. baumannii* FADDI-AB143 (polymyxin B MIC = 16 mg/L). Aliquots (∼20 µL) of early log-phase bacterial suspensions were inoculated into sterile 50 mL polypropylene

tubes (Greiner Bio-one, Frickenhausen Germany) containing 20 mL of Cation-Adjusted Mueller-Hinton Broth (CAMHB), and together with different compositions of polymyxin B and/or silver NPs. After the incubation for 24 h at 37 °C, bacterial colonies were counted using a ProtoCOL automated colony counter (Synbiosis, Cambridge, United Kingdom). The lower limit of detection was 20 CFU/mL.

Table 2.1. The antimicrobial activity of polymyxin B and silver NPs alone and in combination

Bacterial Isolate		MIC (mg/L)				
		MIC Polymyxin B	MIC Silver NPs	FIC Polymyxin B	FIC Silver NPs	FIC[a]
		Polymyxin-resistant isolates				
P. aeruginosa	FADDI-PA069 n/m	256	4	32	1	0.375
	FADDI-PA063 m	256	4	32	0.5	0.25
	FADDI-PA064 n/m	>128	16	32	4	0.375
	FADDI-PA065 m	128	2	16	0.5	0.375
	FADDI-PA066 n/m	64	8	2	2	0.28
	FADDI-PA067 (S) n/m	16	16	2	4	0.375
	FADDI-PA006 m	8	2	1	0.5	0.375
	FADDI-PA068 n/m[a]	64	2	16	1	0.75
	FADDI-PA072 n/m	64	2	4	1	0.56
	FADDI-PA070 n/m	64	8	1	4	0.52
	FADDI-PA014 n/m	4	8	1	1	0.375
	FADDI-PA016 n/m	>128	16	128	2	1
		Polymyxin-susceptible isolates				
	FADDI-PA018 n/m	2	8	0.5	4	0.75
	FADDI-PA020 m	2	8	0.5	4	0.75
	FADDI-PA011 n/m	2	8	0.25	4	0.625
	FADDI-PA005 n/m	2	8	0.5	4	0.75
	FADDI-PA012 m	1	8	0.25	4	0.625
	FADDI-PA015 n/m	1	8	0.125	4	0.625
	FADDI-PA010 n/m	1	8	0.25	4	0.75
	FADDI-PA007 n/m	1	4	0.5	1	0.75
	FADDI-PA017 n/m	1	8	0.25	4	0.75
	FADDI-PA002 m	0.5	4	0.0625	2	0.625
	FADDI-PA031 m	1	2	0.5	1	1
	FADDI-PA019 m	1	4	0.5	2	1
	FADDI-PA008 (L)[b] n/m	1	8	1	8	>1
	FADDI-PA009 (S)[b] n/m	1	8	0.5	4	1
	ATCC 27853	1	4	0.5	2	1
	FADDI-PA013 m	1	4	0.5	2	1
	GFP PA 01	2	4	1	2	1
	FADDI-PA021 m[a]	0.5	1	0.5	1	>1

H. influenzae	FADDIHI003	64	4	16	1	0.5
	FADDI-HI001	32	4	8	2	0.75
B. cepacia	FADDI-BC003	>128	4	256	4	>1
	FADDI-BC001	>128	4	256	4	>1
	FADDI-BC002	>128	4	256	4	>1
B. pseudomallei	BP MSHR1814	>128	8	128	4	1
	BP MSHR3036	>128	8	128	4	1
	BP MSHR912 WGS	>128	8	128	4	1
		Polymyxin-resistant isolates				
A. baumannii	FADDI-AB143	16	16	2	2	0.375
	FADDI-AB148	8	16	4	2	0.375
	ATCC 19606 R	64	4	16	2	0.75
	FADDI-AB060	64	8	16	2	0.5
	FADDI-AB144	8	2	2	0.5	0.5
		Polymyxin-susceptible isolates				
	FADDI-AB150	2	16	0.5	8	0.75
	FADDI-AB151	2	16	0.25	8	0.625
	ATCC 19606	1	4	0.25	1	0.5
	FADDI-AB156	2	8	1	4	1
	FADDI-AB029	1	16	0.5	8	1
	FADDI-AB136	0.5	16	0.5	16	>1
		Polymyxin-resistant isolates				
K. pneumoniae	FADDI-KP027	128	16	1	4	0.26
	FADDI-KP055	64	16	8	4	0.375
	FADDI-KP060	64	8	8	2	0.375
	FADDI-KP058	64	16	2	4	0.28
	FADDI-KP003	64	16	2	4	0.28
	FADDI-KP057	128	16	4	8	0.53
	FADDI-KP028	128	16	2	8	0.52
	FADDI-KP012	32	8	1	4	0.53
	FADDI-KP059	32	8	1	4	0.53
	FADDI-KP062	16	2	2	1	0.625
	FADDI-KP005	4	16	4	16	>1
		Polymyxin-susceptible isolates				
	ATCC 13883	1	2	0.25	1	0.75
	FADDI-KP001	1	16	1	16	>1
		Polymyxin-resistant isolates				
S.maltophilia	FADDI-SM001	4	4	1	0.5	0.375
		Polymyxin-susceptible isolates				
	FADDI-SM002	2	4	1	0.5	0.625
	FADDI-SM006	1	8	0.5	1	0.625
	FADDI-SM004	2	8	1	4	1
	FADDI-SM003	1	16	0.5	8	1
	FADDI-SM007	1	8	1	8	>1
	FADDI-SM008	1	8	0.5	4	1
	FADDI-SM005	0.25	8	0.25	8	>1

Notes: [a]FIC = MIC polymyxin B in combination/polymyxin B MIC alone + MIC silver NPs in combination/silver NPs MIC alone); Synergism FIC < 0.5; Addition FIC = 0.5 – 1.0; Indifference FIC = 1 – 4; Antagonism FIC ≥ 4 (43).

Table 2.2 Antimicrobial susceptibility of *P. aeruginosa* isolates from CF patients.[a]

	Antimicrobial resistance[c, d]									Patient information		
P. aeruginosa strain[b]	PMB	Azt	Cef	Cip	Tic	Mer	Pip	Gen	Tob	Sex	Age	Date collected
FADDI-PA020 m	S	S	S	S	S	-	S	R	S	M	17	19/04/1999
FADDI-PA066 n/m	R	R	R	I	R	-	S	R	R	M	17	19/04/1999
FADDI-PA070 n/m	R	S	R	S	R	-	S	R	R	M	11	19/04/1999
FADDI-PA065 m	R	R	R	S	R	R	S	R	R	M	10	12/04/1999
FADDI-PA064 n/m	R	R	R	S	R	R	R	R	I	M	10	12/04/1999
FADDI-PA021 m	S	S	S	S	S	-	S	R	R	F	16	13/04/1999
FADDI-PA068 n/m	R	R	R	S	R	-	R	R	R	F	16	13/04/1999

Notes: [a]Data were first reported, and reproduced with permission from [23], J. Li, *et al*., *In vitro* pharmacodynamic properties of colistin and colistin methanesulfonate against *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. *Antimicrob. Agents Chemother.* 45, 781 (2001). © 2001, American Society for Microbiology. [b] n/m, non-mucoid; m, mucoid. [c] PMB, polymyxin B; Azt, aztreonam; Cef, ceftazidime; Cip, ciprofloxacin; Tic, ticarcillin; Mer, meropenem; Pip, piperacillin; Gen, gentamicin; Tob, tobramycin. [d] R, resistant; S, susceptible; I, intermediate susceptible.

2.2.4 Nitrocefin assay

The OM permeabilizing activity of the antibiotics was assessed by the β-lactamase nitrocefin assay (398). Briefly, the β-lactamase positive *P. aeruginosa* strain FADDI-PA066 (*P. aeruginosa* strains were tested for β-lactamase production using nitrocefin disc, Sigma-Aldrich, Australia) was sub-cultured on a nutrient agar at 35 °C overnight. The culture was grown to exponential phase and harvested by centrifugation (10,000× g; 10 min). The pellet was washed twice in PBS, pH 7.2 and resuspended in PBS to an OD = 0.50. Nitrocefin was prepared to a concentration of 2 mM in DMSO. The assay was performed in 96-well plates. The 200 µL reaction mixture consisted of 100 µL bacterial suspension, 96 µL drug (2 mg/L polymyxin B or 4 mg/L silver NPs alone or in combination) and 4 µL of 2 mM nitrocefin solution. The absorbance (OD 495 nm) was kinetically measured at room temperature for every minute. The data points are reported as the mean of four independent measurements.

2.2.5 Detection of cellular reactive oxygen species (ROS) production

ROS production in exponential phase (OD 600 nm = 0.1) *P. aeruginosa* FADDI-PA066 cells following antibiotic treatment were measured using the oxidative stress sensitive dye CellROX Green (Lifetechnologies, Australia). CellROX Green fluorescence emission was measured at an excitation wavelength of 485 nm on a Cary Eclipse Fluorescence spectrophotometer (Varian,

Mulgrave, Victoria, Australia) set. The emission spectrum was collected between 490–600 nm and slit widths were set to 5 nm for both the excitation and emission monochromators.

2.2.6 Confocal and electron microscopy imaging methods

Scanning and TEM imaging of *P. aeruginosa* FADDI-PA066 cells treated with polymyxin B (2 mg/L) or silver NPs (4 mg/L) alone or in combination, were performed as we have previously described in detail (399). Confocal imaging of the release of cytosolic green fluorescence protein (GFP) release was performed using the *P. aeruginosa* strain Pa AH298-GFP which expresses GFP under the control of the growth rate dependent $rrnBp_1$ promoter, kindly provided by Professor Philip S. Stewart (400). *Pa* AH298-GFP cells were grown on chamber slides and treated with polymyxin (2 mg/L) or silver NPs (4 mg/L) alone or in combination for 1 h at 37 °C. Cell morphology was assessed using an LSM780 confocal microscope (Zeiss) equipped with a 63× oil objective, excitation/emission filters used were Ex/Em 475/520–560 nm.

2.2.7 Artificial sputum media (ASM) Biofilm Assay

The ASM assays were performed with *P. aeruginosa* strain FADDI-PA065 in a 24-well plate format. ASM culture medium ideal mimics the components of CF patient sputum, and includes egg yolk, amino acids, mucin and DNA combined in ratios as described in detail by Kirchner *et al*., prepared fresh and filter sterilized (398, 401). *P. aeruginosa* strain FADDI-066 was sub-cultured on a nutrient agar and incubated at 37 °C overnight and one colony was randomly selected and grown overnight in 10 mL CAMBH at 37 °C. The overnight culture was diluted in CAMBH to an OD of 0.05 ± 0.01 and then further diluted 1:100 in fresh ASM. A total volume of 1.8 mL was added to each well. Plates were secured with parafilm and incubated for 3 days under aerobic conditions at 37 °C in which the *P. aeruginosa* biofilms were developed. After 3 days polymyxin B (2 mg/L) or silver NPs (4 mg/L), alone or in combination were added and further incubated for 24 hours. For each antibiotic concentration four replicates were conducted. After the 24 h incubation, biofilms were disrupted with 100 µL of 100 mg/mL, cellulase (diluted

in 9.6 g/L citrate; pH adjusted to 4.6 with NaOH) and incubated for 1 h at 37 °C. Biofilms were further manually disrupted by pipetting. To determine the cell viability 100 µL of 0.02% (v/v) resazurin was added to each well and further incubated for 2 h at 37 °C. Fluorescence (F) of each well was measured using an ENVISION plate reader (PerkinElmer, Australia) set at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Fluorescence was corrected by the subtraction of the background noise. The cell viability was calculated as (mean F of treated biofilms/mean F untreated control) ×100%.

2.3 Results and Discussion

2.3.1 Antibacterial synergy testing of the polymyxin B-silver NP combination sgainst Gram-negative CF isolates

We have employed polymyxin B for this in vitro study as the clinically used prodrug form of colistin, CMS, is an inactive prodrug and would result in conversion to colistin in the assay buffers (402). Moreover, in patients, polymyxin B has a better pharmacokinetic/ pharmacodynamic profile compared to CMS (146). The MICs for polymyxin B and silver NPs alone, and in combination are presented in **Table 2.1**. The broth microdilution checkerboard method results showed the combination displayed synergism against 7 out of the total 12 polymyxin-resistant CF isolates of *P. aeruginosa* examined. The combination displayed additive activity (based on FICs) against the remaining 5 *P. aeruginosa* polymyxin-resistant CF isolates. Similarly, additivity was observed against all of the 16 polymyxin-susceptible *P. aeruginosa* CF isolates tested. The combination displayed synergism against 2 out of a total 5 polymyxin-resistant *A. baumannii* isolates and additivity against the remaining 3 isolates. The combination displayed additive activity against 5 of the 6 *A. baumannii* polymyxin-susceptible isolates tested and indifference against one. Notably, the combination displayed better activity against the polymyxin-susceptible strain *A. baumannii* ATCC 19606 (FIC = 0.5) than it paired polymyxin-resistant *A. baumannii* ATCC19606R (FIC = 0.75). We have previously reported a unique polymyxin resistance mechanism employed by *A. baumannii* 19606R which involves the loss of LPS from the OM (94). Silver ions have been reported to increases membrane

permeability through disruption of disulfide bond formation and misfolded protein secretion (386). Considering that the silver NPs alone showed similar bactericidal activity against both the polymyxin-susceptible ATCC19606 and resistant *A. baumannii* ATCC19606R strains (MIC 4 mg/L for both isolates); hence, it is very unlikely LPS plays a key role in the membrane disrupting activity of silver NPs (386). The combination was ineffective, showing additivity or indifference, against the 6 *B. cepacia* and *B. pseudomallei* strains tested as this species is intrinsically highly resistant to polymyxins (All MICs > 128 mg/L), due to the modification of the phosphate groups of the lipid A (403, 404). Nevertheless, the silver NPs alone exhibited moderate bactericidal activity against *B. cepacia* and *B. pseudomallei* with MICs of 4 and 8 mg/L, respectively. The combination of polymyxin B and silver NPs was synergistic against 5 out of the total 11 polymyxin-resistant *K. pneumoniae* isolates and additive against the remaining 6. The combination did not show synergy against the polymyxin-susceptible *K. pneumoniae* ATCC 13883 and FADDI-KP001 isolates. Similarly, the combination was not synergistic against polymyxin-susceptible *S. maltipholia* isolates, however, synergisim was observed against the polymyxin-resistant *S. maltophilia* FADDI-SM001 isolate. Collectively, the antibacterial testing data indicated the combination of polymyxin B with silver NPs at clinically relevant concentrations display synergy against polymyxin-resistant isolates *P. aeruginosa, A. baumannii, K. pneumoniae* and *S. maltophilia*. Notably, the combination achieved 42% synergy coverage across a total of 36 polymyxin-resistant isolates tested, which is most encouraging. The less than complete coverage is likely due to variability in susceptibility to the antibacterial mechanisms involved across the Gram-negative isolates. Bacteria are less likely to develop resistance against silver as due to the broad range of cellular targets silver ions attack (386). Notwithstanding, microbial resistance to silver ions has been reported for *Cryptococcus neoformans* and *Salmonella*, where resistance is associated with an overexpression of copper-related efflux pumps (405, 406). We would only go as far to speculate that these effects may be associated with the different levels of aminoarabinose modified lipid A (the primary mechanism that confers the level of polymyxin B resistance in *P. aeruginosa*

CF isolates) (394) in the OM of each isolate. To this end, further studies which are beyond the scope of the present report, are currently underway to elucidate the lipid A levels in relation to these complex killing effects.

2.3.2 Anti-pseudomonal killing kinetics of the polymyxin B-silver NP combination

The synergistic killing activity of the combination in static time-kill studies was assessed against two polymyxin-resistant *P. aeruginosa* CF isolates, FADDIPA066 (polymyxin B MIC = 64 mg/L; silver NP MIC =8 mg/L) and FADDI-PA067 (polymyxin B MIC =16 mg/L; silver NP MIC = 16 mg/L) **(Figure 2.1(A))**; one polymyxin-resistant *A. baumannii* isolate, FADDI-AB0143 (polymyxin B MIC= 16 mg/L; silver NP MIC= 16 mg/L) and one polymyxin-resistant *K. pneumoniae* isolate, FADDIKP027 (polymyxin B MIC = 128 mg/L; silver NP MIC =16 mg/L) **(Figure 2.1A)**. Monotherapy with polymyxin B exhibited no antibacterial activity against the two *P. aeruginosa* isolates up to 24 h and the bacterial killing curve was essentially indistinguishable from that of the controls **(Figure 2.1A)**. In contrast, the polymyxin B -silver NPs combination (polymyxin B 2 mg/L and silver NPs 4 mg/L) demonstrated synergism in the static time-kill studies against the two *P. aeruginosa* isolates, as indicated by ≥ 2 $\log_{10}$ reduction in CFU/mL, compared to polymyxin B monotherapy. Notably, the combination showed time-dependent bactericidal activity during 24 h against both *P. aeruginosa* CF isolates. Monotherapy with the silver NPs showed some killing during 24 h. Neither silver NPs nor polymyxin B alone demonstrated any killing activity against the *A. baumannii* FADDI-AB0143 or *K. pneumoniae* FADDI-KP027 **(Figure 2.1A)**. However, in combination produced significant killing (>2 $\log_{10}$ reduction) was evident from 2 to 8 h against both these isolates. However, unlike the *P. aeruginosa* isolates, re-growth at 24 h was evident for both *A. baumannii* FADDIAB0143 and *K. pneumoniae* FADDI-KP027. Overall, the data from the time-kill studies are consistent with the findings from the checkerboard experiments, with synergistic killing against a sub-set of polymyxin-resistant clinical isolates of *P. aeruginosa, A. baumannii* and *K. pneumoniae*.

2.3.3 Anti-pseudomonal activity of the polymyxin B-silver NP combination in sputum

In order to test the antibacterial activity of the combination under more CF relevant conditions, an ASM antibacterial assay was employed **(Figure 2.1B)**. The ASM findings showed that the combination was very effective (producing a ~85% reduction in bacterial cell viability) in sputum-like conditions under planktonic growth conditions against the polymyxin-resistant *P. aeruginosa* CF isolate FADDI-PA066 (polymyxin B MIC = 64 mg/L; silver NP MIC = 8 mg/L). In comparison, polymyxin B alone at 2 mg/L was completely ineffective, whereas the silver NP alone at 4 mg/L managed to reduce the bacterial cell viability by ~50%. However, in ASM under biofilm growth conditions, the combination and the individual treatments were inactive, possibly due to the inability of the agents to penetrate the robust biofilm matrix of extracellular polymeric substance (407).

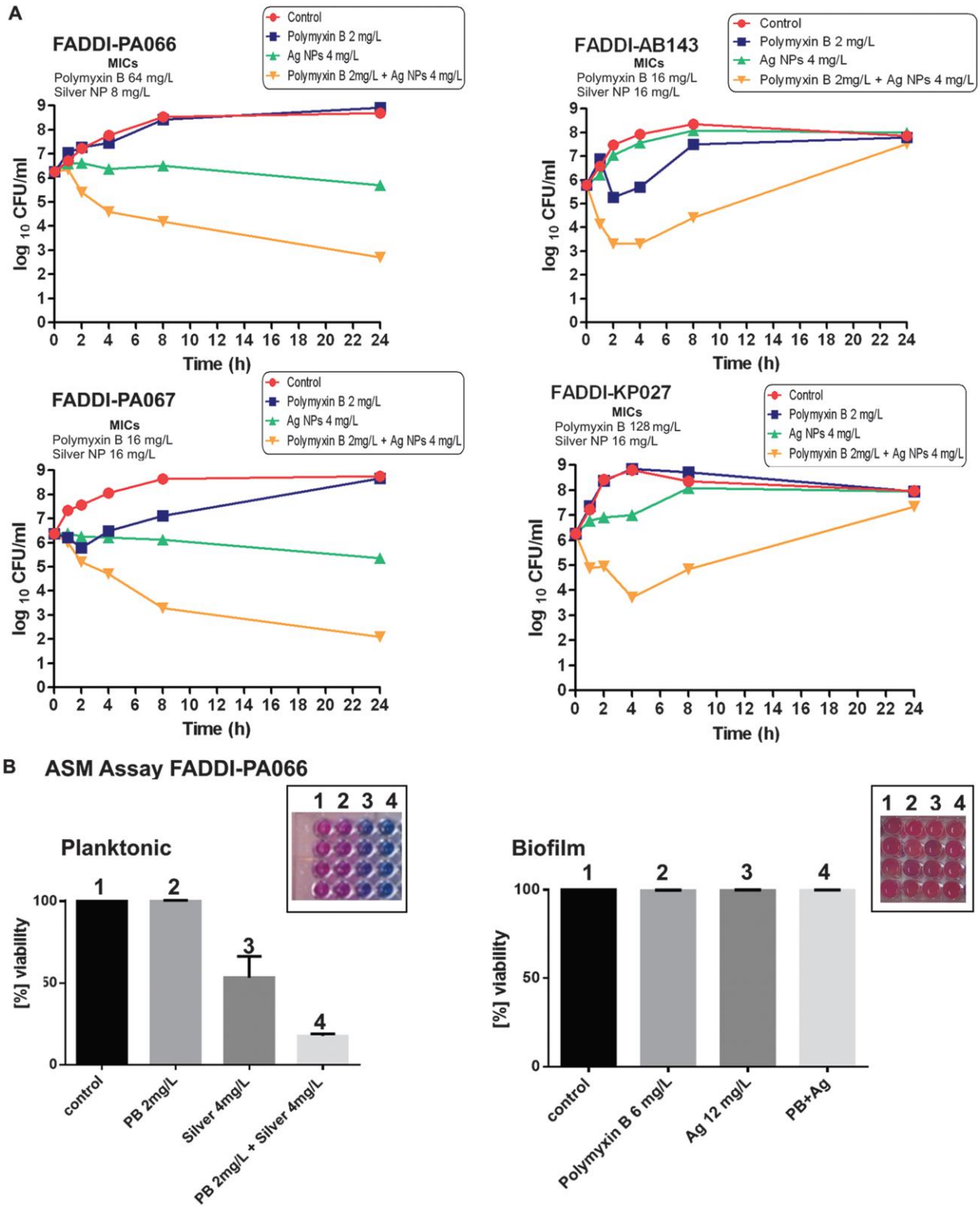


Figure 2.1(A) Time-kill curves for the combination of polymyxin B and silver NPs against polymyxin-resistant non-mucoid *P. aeruginosa* CF isolates FADDI-PA066 and FADDI-PA067, *A. baumannii* FADDI-AB143 and *K. pneumoniae* FADDI-KP027. **(B)** Antimicrobial activity of the combination against *P. aeruginosa* FADDI-PA066 in artificial sputum (ASM) media under planktonic and biofilm growth conditions. Data points are plotted as the mean±SD of four independent measurements. Both the combination and silver NPs alone induced significant reduction in cell viability under planktonic grwoth condition. The top right-hand corner insets graphics in each bar graph show the raw data from the

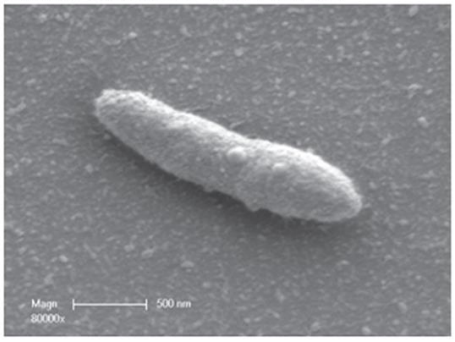
resazurin fluorescence plate assay; the numbering 1–4 corresponds to the numbering for each condition in the adjacent bar graph. The replicates for each condition are organized vertically in the 96-well plate.

2.3.4 Imaging the Gram-negative outer membrane permeabilizing activity of the polymyxin B silver NP combination

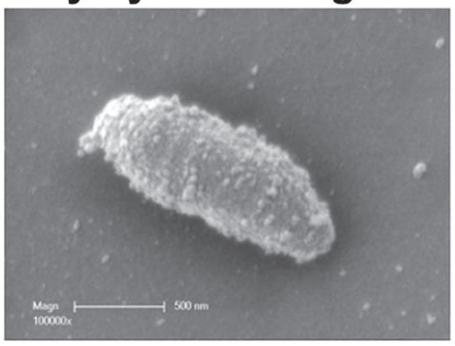
Scanning and transmission electron (SEM/TEM) microscopy imaging of *P. aeruginosa* FADDI-PA066 cells treated with the combination showed the OM structure was extensively disrupted in a distinctive fashion **(Figure 2.2)**. The morphological changes to the OM observed following treatment with the combination were very different from the changes seen with polymyxin B (2 mg/L) treatment alone where a wide-spread blebbing effect was observed; and treatment with silver NPs (4 mg/L) alone where large OM protrusions were observed. Our SEM findings with polymyxin B treatment alone are consistent with the literature reports (408). Similarly, confocal imaging revealed that the combination is more effective at producing OM damage that leads to cytosolic GFP release from a GFP expression strain of *P. aeruginosa* than either silver NP or polymyxin B treatment *per se* **(Figure 2.3)**.

Scanning electron microscopy FADDI-PA066

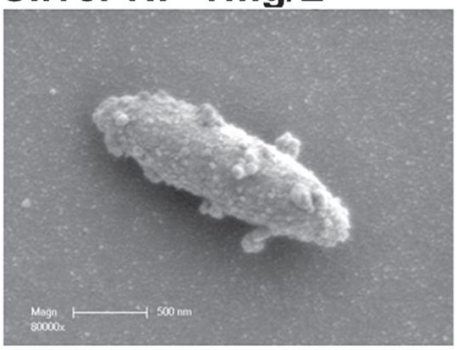
Untreated control



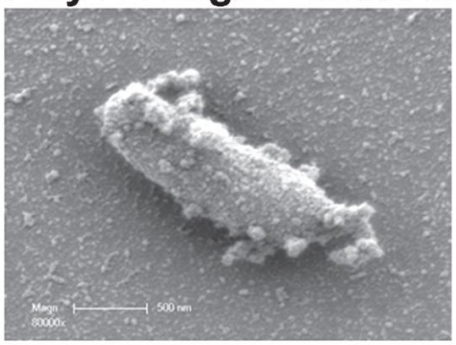
Polymyxin B 2 mg/L



Silver NP 4 mg/L

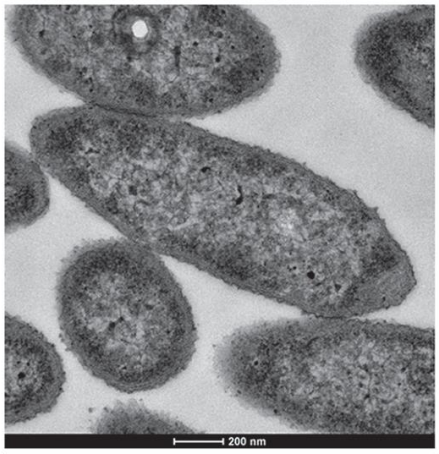


Poly B 2 mg/L +Silver NP 4 mg/L

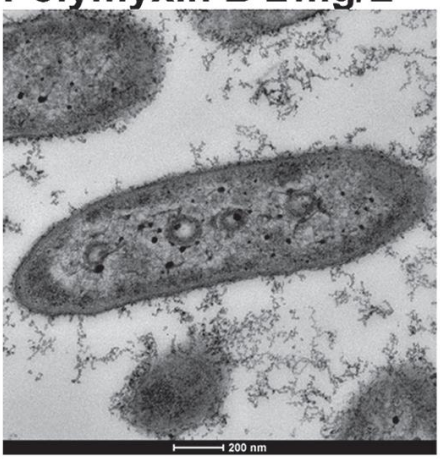


Transmission electron microscopy FADDI-PA066

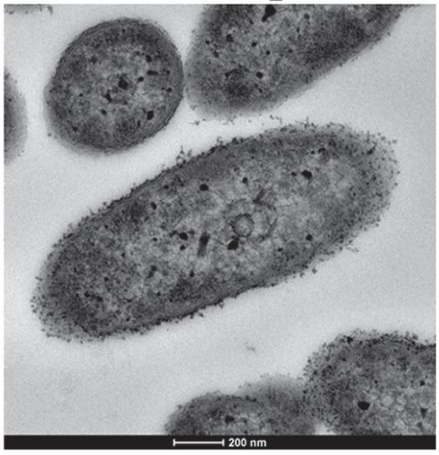
Untreated control



Polymyxin B 2 mg/L



Silver NP 4mg/L



Poly B 2 mg/L +Silver NP 4mg/L

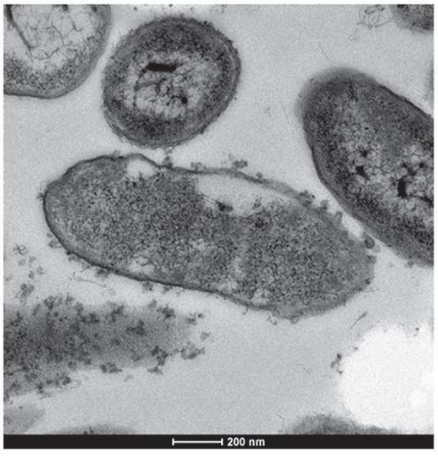


Figure 2.2 Electron microscopy images of *P. aeruginosa* CF isolate FADDI-PA066 (polymyxin B MIC = 64 mg/L; silver NP MIC = 8 mg/L) treated with the polymyxin B-silver NP combination. SEM imaging of bacterial cells treated with both polymyxin B (2 mg/L) alone and silver NPs (4 mg/L) alone showed shortening of the cells, and enlargement of the cells possibly due to bleb-like projections on the cell wall.

P. aeruginosa loss of cytosolic GFP

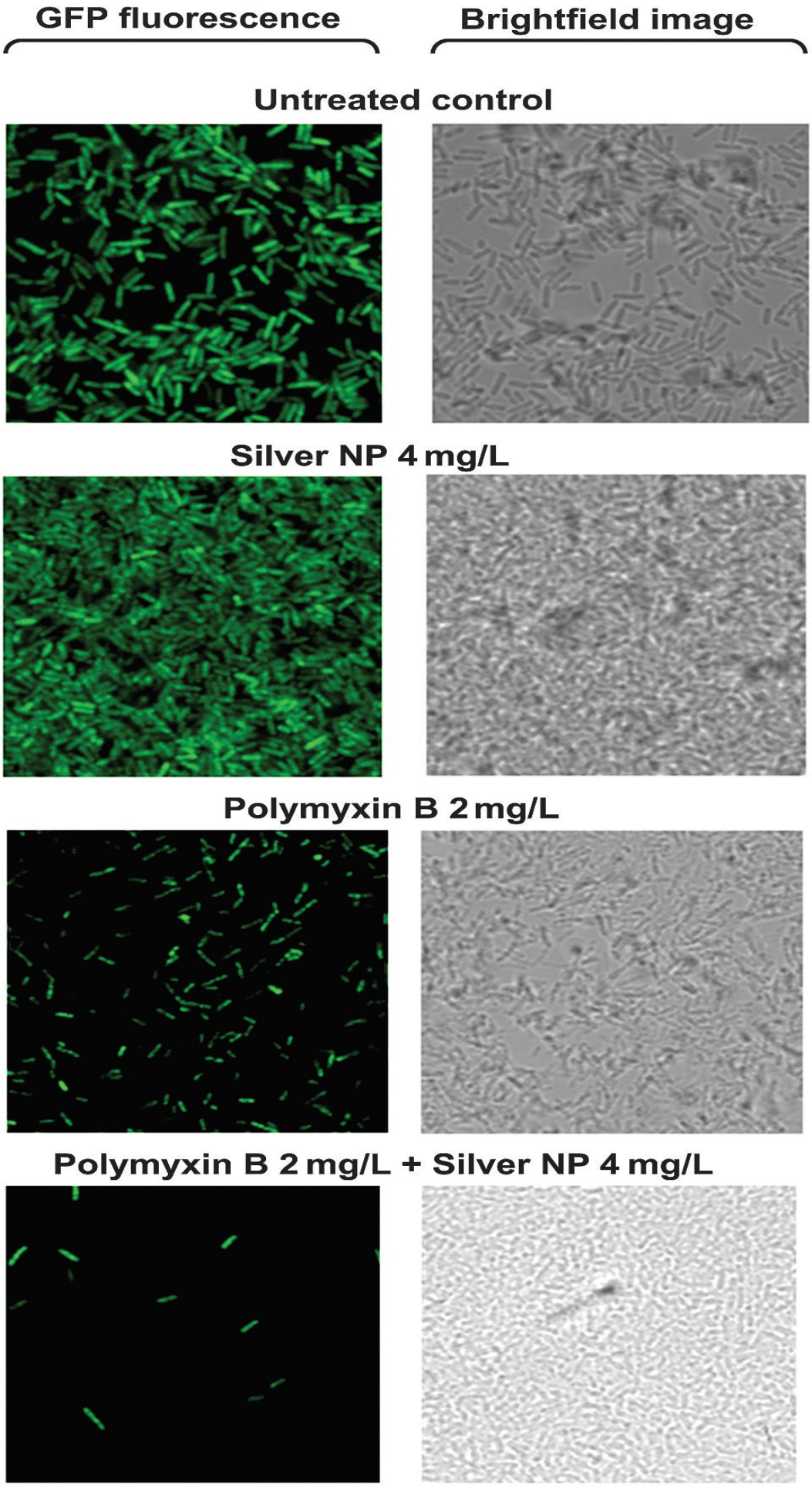


Figure 2.3 Confocal microscopy images of cytosolic GFP release from *P. aeruginosa* AH298-GFP (polymyxin B MIC = 2 mg/L; silver NP MIC = 4 mg/L) treated with the polymyxin B-silver NP combination. The initial CFU was same for the four growth conditions.

2.3.5 The polymyxin B-silver NP combination induces increased nitrocefin uptake across the outer membrane and production of reactive oxygen species

Additional evidence that the combination causes superior damage to the OM than treatment with either compound alone, comes from the nitrocefin assay data with *P. aeruginosa* FADDI-PA066 cells. The assay measures the periplasmic β-lactamase activity using nitrocefin, a chromogenic cephalosporin substrate analogue, which normally penetrates the OM very slowly. In the presence of OM permeabilizing agent nitrocefin can more easily permeate the OM barrier and enter the cell where it is converted to its coloured form by β-lactamase. The polymyxin B-silver NP combination was more effective at permeabilizing the OM of the FADDI-PA066 cells than treatment with either compound per se, as seen from the increased β-lactamase mediated conversion of nitrocefin **(Figure 2.4A)**. ROS production is purported to be a common bacterial killing mechanism for antibiotics (409). **Figure 2.4B** shows that the combination induced a greater increase of ROS production in *P. aeruginosa* FADDI-PA066 cells compared to treatment with each agent alone, which was seen as an increase in the fluorescence emission of the oxidative stress sensitive dye CellROX Green.

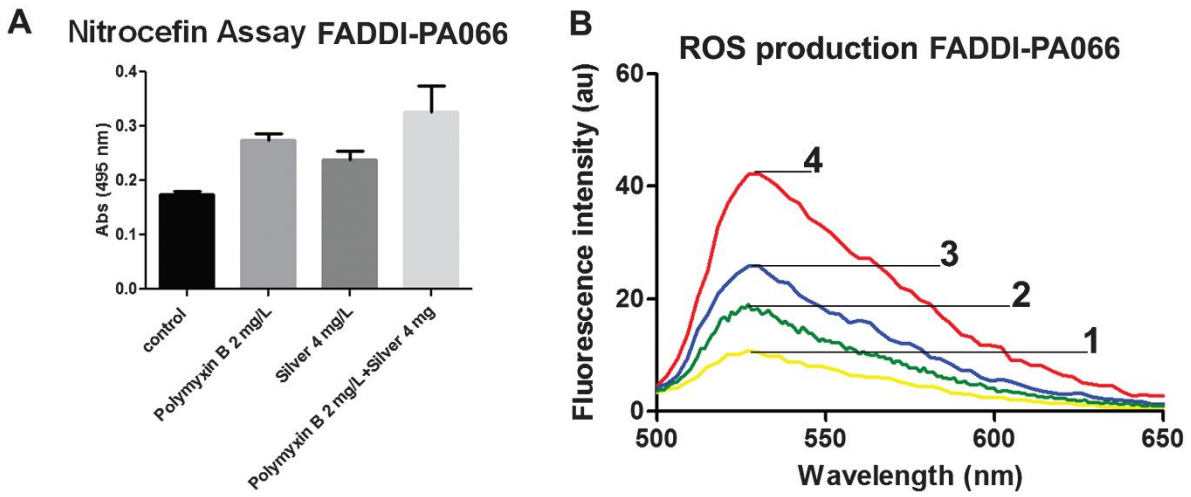


Figure 2.4 (A) OM permeabilizing activity of polymyxin B and silver NP alone and in combination against *P. aeruginosa* FADDI-PA066 measured by the nitrocefin assay. The β-lactamase activity was measured by the nitrocefin assay for 140 sec. The results are presented the mean of four independent measurements ± standard deviation. The combination of polymyxin B and silver NP significantly increased the permeability of *P. aeruginosa* OM. (**B)** ROS production in *P. aeruginosa* FADDI-PA066 cells following treatment measured by the increased fluorescence emission of the oxidative stress sensitive dye CellROX Green. The combination of polymyxin B and silver NP significantly increased the release of ROS (4). Treatments: 1, Untreated cells; 2, polymyxin B 2 mg/L; 3, Silver NP 4 mg/L; 4, Combination of polymyxin B 2 mg/L+ silver NP 4 mg/L.

2.4 Conclusions

The antibacterial synergistic activity of polymyxin B in combination with silver NPs is appreciable in the view that both agents cause damage to the Gram-negative OM via different mechanisms (386). Polymyxins act via direct interaction with the lipid A component of the LPS which leads to a disruption of the barrier function of the Gram-negative OM (15). Our group has also shown that polymyxin B may act via a secondary mechanism through the inhibition of the type II NADH-quinone oxidoreductase inner membrane respiratory enzyme (410). Although the precise mechanisms of action of silver NPs, is not fully understood it is known they compromise the integrity of the OM through disruption of disulfide bonds within inner membrane respiratory enzymes, which in turn leads to the production of damaging ROS (386). Therefore, the underlying mechanistic basis for the bactericidal synergy of the combination may involve their combined membrane permeabilising activity and the respiratory chain poisoning

activity of each agent. The findings from the present study suggest that the combination of polymyxins with silver NPs could potentially prove useful in a clinical setting for the treatment of MDR polymyxin-resistant *P. aeruginosa* lung infections in CF patients. Given that silver NPs and polymxyins have been shown to be relatively non-toxic in lung tissues (393, 411, 412), there is a great potential to combine silver NPs with polymyxins and develop a new nebulizer formulation for CF patients.

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Chapter 3: Comparative analysis of the sub-proteome of outer membrane vesicles from a paired polymyxin-susceptible and -resistant *Klebsiella pneumoniae*

Abstract

Gram-negative bacteria constitutively secrete OMVs as a cargo for numerous bacterial heinous factors such as virulence factors and antibiotic resistance determinants. This study aimed to investigate the effect of polymyxin B treatment on the OMVs proteome from paired polymyxin-susceptible and -resistant *K. pneumoniae* ATCC 700721 isolates.

Subcellular localisation prediction revealed that the OMVs protein composition of both polymyxin-susceptible and –resistant strains predominantly consisted of cytoplasmic and periplasmic proteins. However, the comparative analysis of these proteins showed that polymyxin B treatment of both strains significantly upregulate the cytoplasmic proteins and downregulate the inner membrane, periplasmic and OMPs. Comparative profiling of the OMVs proteome of each strain revealed that different bacterial metabolic pathways were overrepresented in the OMVs of sensitive strain, including lipopolysaccharide and peptidoglycan biosynthesis pathways. In comparison, in the resistant strain, OMVs proteins from QS, phosphotransferase system, RNA degradation and nucleotide excision repair pathways were significantly over-expressed in response to polymyxin B treatment. Moreover, translation, ribosomal structure and biogenesis, Aminoacyl-tRNA biosynthesis, amino acids biosynthesis, UDP-amino sugar and nucleotides pathways were upregulated in the OMVs of both treated strains. Whereas, ABC transporters, two-component system, cationic antimicrobial peptide resistance and beta-lactam resistance pathways were significantly underrepresented in OMVs of both strains following polymyxin B treatment. These findings shed new light on the selective sorting of OMVs proteome accompanying polymyxin B treatment. Putatively, these proteomic changes may serve to make the *K. pneumoniae* OM and OMVs invincible to polymyxin attack.

Key words: Outer membrane vesicles, proteomics, Gram-negative, polymyxin-resistance, *Klebsiella pneumoniae*.

3.1 Introduction

Extremely-drug resistant (XDR) *K. pneumoniae* has emerged as one of the most deadly Gram-negative 'superbugs' (413-415), and is responsible for numerous lethal nosocomial and community outbreaks (416). Worryingly, *K. pneumoniae* infections can have up to a 50% mortality rate if effective antibiotic treatment is not promptly administered (417). Resistance against the first-line carbapenems in *K. pneumoniae* was firstly reported in 1996 in the USA and has spread globally (417, 418). In 2008, bla_{NDM-1} which encodes the class B NDM-1 that inactivates carbapenems was first detected in a Swedish patient of that had contracted an infection in India (419). The polymyxin lipopeptide antibiotics (i.e. colistin and polymyxin B) are increasingly used as the last-line therapy against these problematic XDR *K. pneumoniae* strains (15). Indeed, considerable *in vitro* activity against *K. pneumoniae* has been demonstrated (136); 98.2 % of general clinical strains of *K. pneumoniae* are susceptible to polymyxin B and colistin (420-425). Notwithstanding, XDR strains which are resistant to polymyxins have recently emerged (426, 427), which underscores the need for a greater effort for further investigation of polymyxin resistance mechanism(s) in *K. pneumoniae*.

The Gram-negative OM constitutes a permeability barrier to various noxious substances for the bacterial cell, such as antimicrobial drugs (156, 428). This complex asymmetrical structure comprises an inner phospholipid leaflet, as well as an outer leaflet that predominantly contains LPS, proteins and lipids. Additionally, *K. pneumoniae* commonly expresses a capsular polysaccharide that coats the OM, the expression levels of which have been related to decreased levels of polymyxin susceptibility (429-432). The antimicrobial killing action of the polymyxins is mediated through direct interaction with the lipid A component of the LPS which leads to a disruption of the selective permeability of the OM barrier (15). The cationic L-α,γ-Dab residues of the polymyxin molecule mediate an electrostatic attraction to the negatively charged lipid A phosphate groups, displacing the divalent cations (Mg^{2+} and Ca^{2+}) (15). This displacement leads to the disorganisation of the LPS layer, which enable the insertion of polymyxin molecule into the fatty acyl layer of the OM (433). Polymyxin resistance in *K. pneumoniae* primarily involves

the overexpression of capsular polysaccharide expression and the modification of lipid A with 4-amino-4-deoxy-L-arabinose or phosphoethanolamine (173, 429, 432, 434-440). In *K. pneumoniae* the expression of 4-amino-4-deoxy-L-arabinose modifications to the lipid A phosphates is under the control of the two-component regulatory systems [PhoPQ-PmrD]-PmrAB that are activated in response to low pH, low magnesium and high iron (432). Specifically, the PhoP-PhoQ (collectively PhoPQ) system regulates the magnesium regulon, which may activate the resistance to polymyxin B under low magnesium conditions. PmrD, which is a small basic protein, is responsible for the connection of PhoP-PhoQ system. PhoP controls the activation of PmrD, which can then bind to PmrA and prolong its phosphorylation state, eventually activating the expression of the PmrA-PrmB (collectively PmrAB) system to promote resistance to polymyxin. The under-acylation of lipid A increases the polymyxin susceptibility of *K. pneumoniae;* which highlights that the decoration of lipid A with additional fatty acyl chains is important for polymyxin resistance (441, 442). OMPs have also been implicated in OM remodelling that is associated with increased polymyxin susceptibility in *K. pneumoniae* (430).

Gram-negative bacteria can shed components of their OM via OMVs, which are spherical bilayer structures of approximately 20-200 nm in diameter (443). Structurally, these vesicles are built up of OM and periplasmic lipids and proteins (444). Besides, OMVs act as cargo for other bacterial components such as cytoplasmic and inner membrane proteins, genetic materials, virulence factors, metabolites and signalling molecules (275-278). Therefore, these membrane vesicles play a crucial role in bacterial virulence, inflammation, and host immune stimulation, as well as, in the defence mechanisms against antibiotics (190). OMVs protect bacteria from the action of antibiotics either by their contents of antibiotic-degrading enzymes (e.g., β-lactamase and aminoglycosides-inactivating enzymes) or by capturing the membrane-active antibiotics such as polymyxins (190, 291, 313, 332). This highlights the need to understand the compositional differences of the OMV sub-proteome between polymyxin-susceptible and -resistant *K. pneumoniae* isolates. In the present study, we primarily aimed to perform a

comparative analysis of the OMV proteome of paired polymyxin-susceptible and -resistant *K. pneumoniae* ATCC 700721 and to identify key proteins that are selectively packaged from the parent bacteria into the OMV sub-proteome. The garnered data sheds new light on the OMV sub-proteome associated with polymyxin resistance in the problematic Gram-negative human pathogen, *K. pneumoniae*.

3.2 Methods

3.2.1 Materials

Polymyxin B was supplied by Betapharma (Shanghai, China). All chemicals were purchased from Sigma-Aldrich at the highest research grade, ultrapure water was from Fluka (Castle Hill, NSW, Australia), Tris from ICN biochemicals (Castle Hill, NSW, Australia) and the precast-SDS gels were from (NuSep Ltd, Lane Cove, NSW, Australia). Stock solutions of polymyxin B (10 mg/L) were prepared in Milli-Q™ water (Millipore, North Ryde, NSW, Australia) and filtered through 0.22 µm syringe filters (Sartorius, Melbourne, Vic, Australia).

3.2.2 Bacterial isolates and growth conditions

Two *K. pneumoniae* isolates were studied *K. pneumoniae* ATCC 700721 (polymyxin B MIC = 0.5 mg/L) and its paired polymyxin-resistant strain (polymyxin B MIC = >32 mg/L). All bacteria were stored at -80ºC in tryptone soya broth (TSB, Oxoid Australia). Prior to experiments, parent strains were sub-cultured onto nutrient agar plates (Medium Preparation Unit, University of Melbourne, VIC, Australia). Overnight broth cultures were subsequently grown in 5 mL of cation-adjusted Mueller-Hinton broth (CaMHB, Oxoid, West Heidelberg, VIC, Australia), from which a 1 in 100 dilution was performed in fresh broth to prepare mid-logarithmic cultures according to the optical density at 500 nm (OD_{500nm} = 0.4 to 0.6). All broth cultures were incubated at 37°C in a shaking water bath (180 rpm).

3.2.3 Minimum Inhibitory Concentration (MIC) microbiological assay

Resistance to polymyxin B was defined as MICs of ≥8 mg/L (445). MICs were performed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (446). MICs were determined for all isolates in three replicates on separate days using broth microdilution method in cation-adjusted Mueller-Hinton broth (CAMHB) in 96-well polypropylene microtitre plates. Wells were inoculated with 100 µL of bacterial suspension prepared in CaMHB (containing 10^6 colony forming units (CFU) per mL) and 100 µL of CaMHB containing increasing concentrations of polymyxins. The MICs were defined as the lowest concentration at which visible growth was inhibited following 18 h incubation at 37°C. Cell viability was determined by sampling wells at polymyxin concentrations greater than the MIC. These samples were diluted in normal salineand spread plated onto nutrient agar. After incubation at 37°C for 20 h, viable colonies were counted on these plates. The limit of detection was 10 CFU/mL.

3.2.4 Isolation of outer membrane vesicles (OMVs)

Mid-logarithmic cultures (4 L) of each isolate were grown, polymyxin B was added to the culture volume at a final concentration of 2 mg/L, and cell free supernatants were collected through centrifugation (15 min at 10,000 × *g*, 4°C). The OMV containing supernatants were filtered through 0.22-µm membrane to remove any remaining cell debris, concentrated through a tangential filtration concentrator unit (Pall Life Science, Ann Arbor, MI); and collected using 100 kDa Pellicon filtration cassettes (Millipore, Melbourne, Australia). A portion of the supernatant was plated for growth on agar plates overnight at 37°C to make sure that the supernatant was free of bacterial cells. OMVs in the cell-free supernatants were then pelleted down by ultracentrifugation at 150,000 × *g* for 2 h at 4 ◦C in a Beckman Ultracentrifuge (SW28 rotor). Purified OMVs were concentrated and re-suspended in 1 mL of sterile PBS. The protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Melbourne, Australia).

3.2.5 Ultra High Pressure Liquid Chromatography coupled with Mass Spectrometry

For all experiments, an Ultimate3000 ultra high-pressure liquid chromatography (UHPLC) system (Dionex, Castle Hill, Sydney) was used equipped with a ternary low pressure mixing gradient pump (LPG-3600), a membrane degasser unit (SRD-3600), a temperature controlled pulled-loop autosampler (WPS-3000T) and a temperature controlled column oven with flow manager (FLM-3100). The UHPLC experiments were performed using the "pre-concentration" set-up under the following conditions: Nano-column C_{18} PepMap100, 75 µm ID × 150 mm, 3 µm, 100 Å; mobile phase A: 99.9% water + 0.1% FA (v/v, formic acid); mobile phase B: 20/80 water/acetonitrile (ACN) (v/v) + 0.08 % formic acid; flow rate nano-column, 400 nL/min; gradient, 2-40% B over 45 min, 90% B for 5 min, 4% B for 30 min; loop size, 5 µL; injection volume, 4 µL (FullLoop) by User Defined Program. The oven temperature was set to 35°C. For the identification of peptides, CID experiments were performed using an AmaZon ETD Ion Trap (Bruker Daltonik GmbH, Australia) equipped with an online-nano-sprayer spraying from a 0.090 mm i.d. and 0.02 mm i.d. fused silica capillary. Fine tuning was achieved using the smart parameter setting option (SPS) for 900 *m/z*, compound stability 60%, and trap drive level at 100% in normal mode resulted in the following mass spectrometric parameters: dry gas temperature, 180°C; dry gas, 4.0 L min^{-1}; nebulizer gas, 0.4 bar; electrospray voltage, 4500 V; high-voltage end-plate offset, -200 V; capillary exit, 140 V; trap drive, 57.4; funnel 1 in 100 V, out 35 V and funnel 2 in 12 V, out 3.3 V; ICC target, 500000; maximum accumulation time, 50 ms. The sample was measured with the Enhanced Scan Mode at 8100 *m/z* per second (which allows monoisotopic resolution up to four charge stages), polarity positive, scan range from *m/z* 100-3000, 5 spectra averaged and rolling average of 2. Acquired tandom mass spectra were processed in Data Analysis 4.0; deconvoluted spectra were further analyzed with BioTools 3.2 software and submitted to Mascot database search (Mascot 2.2.04, Swissprot database. The species subset was set at *K. pneumoniae,* parent peptide mass tolerance +/- 0.4 Da, fragment mass tolerance +/- 0.4 Da; enzyme specificity trypsin with 2 missed cleavages considered. The

following variable modifications have been used: Deamidation (NQ) and Oxidation (M) and carrbomidiomethyaltion (C).

3.2.6 Bioinformatics

The derived MS datasets on the 3D-trap system were combined into protein compilations using the Protein Extractor functionality of Proteinscape 2.1.0 573 (Bruker Daltonics, Bremen, Germany), which conserved the individual peptides and their scores, while combining them to identify proteins with much higher significance than achievable using individual searches. To exclude false positive identifications, peptides with Mascot scores below 40 (chosen on the basis of manual evaluation of the MS/MS data of peptides with scores below this number) were rejected. The identified protein sequences were manually validated in BioTools (Bruker Daltonics, Bremen, Germany) on a residue-by residue basis using the raw data to ensure accuracy. In brief, the ion series were inspected to ensure: the peaks being selected were not simply base-line, the accuracy between the residues was less than 0.15 Da and that, preferentially, an overlapping ion series was found, as explained in greater detail in the results section. When run against a reversed database, and the spectra manually interpreted in a similar fashion, we found a false discovery rate of less than 0.1%. Since this strain (strain ATCC 700721 / MGH 78578) has complete genomic map in UniProt (447), the identified proteins were searched in UniProt and crossreferred using MGH 78578 KEGG (448) identifiers to identify the protein name and KEGG IDs. The subcellular localisation of each protein was predicted using PSORTb 3.0 and Cello V2.5 (449, 450). Comparative analysis of significantly changed proteins (fold-change >2 and p-value < 0.05) was performed using MaxQuant (version 1.2.2.5) (451).The protein associated metabolic pathways of deferentially over- and under-represented proteins were highlighted in KEGG mapper.

3.3 Results and Discussion

3.3.1 OMV sub-proteome characterization from polymyxin-susceptible and resistant *K. pneumoniae* isolates

Overall, OMVs proteomics identified 1296 and 1161 differentially represented proteins in at least two of three biological replicates of polymyxin-susceptible *K. pneumoniae* ATCC 700721 and its paired polymyxin-resistant strains, respectively. Comparative analysis displayed that 123 proteins were significantly overrepresented and 109 proteins underrepresented in OMVs of polymyxin B treated polymyxin-susceptible *K. pneumoniae* compared to the untreated control **(Tables 3.1 and 3.2)**. While, in the OMVs of the paired polymyxin-resistant strain, 75 proteins were significantly overrepresented and 37 proteins underrepresented in response to polymyxin B treatment **(Tables 3.3 and 3.4)**.

3.3.2 Subcellular localisation of significantly changed proteins

Subcellular localisation prediction revealed that protein contents of the OMVs from both *K. pneumoniae* strains consisted mostly of cytoplasmic (~48%), inner membrane (~25%) and periplasmic proteins (~18%). Similarly, across both strains the OMV minor protein components consisted of proteins originated from the OM (~7%), and extracellular proteins (~2%). Polymyxin B treatment of polymyxin-susceptible strain significantly increased the number of cytoplasmic proteins (124 out of 141) in its OMVs, whereas of the 57 inner membrane and 34 periplasmic proteins, 56 and 41 were significantly downregulated respectively. Of four OM proteins, two were upregulated and two underrepresented **(Figure 3. 1A)**. Similarly, of the 70 cytoplasmic proteins, 65 were upregulated, and of the 26 periplasmic proteins, 24 were upregulated and two were downregulated in OMVs from the resistant strain in response to polymyxin B treatment, while of the 11 OM proteins, nine were underrepresented and only two proteins were significantly overrepresented **(Figure 3.1B)**. Dutta *et. al*., showed that *S. dysenteriae* type 1 release OMVs which composed of only OM proteins (322). However, our *K. pneumoniae*-derived OMVs contain high numbers of cytoplasmic proteins including the 30S and 50S ribosomal proteins, and tRNA synthetases following polymyxin B treatment of both

susceptible and resistant strains. Moreover, periplasmic proteins thought to be a natural cargo sorted into the OMVs during vesiculation (452). Polymyxin B treatment of both strains remarkably reduced the periplasmic protein contents of the OMVs. Growing evidence suggests that bacterial OMVs is a novel protein secretory mechanism (320, 453). We previously reported that polymyxin B treated *K. pneumoniae* ATCC700721 release OMVs larger in particle size than untreated strains (454). These findings would suggest that polymyxin B treatment significantly alter the selective sorting of OMVs proteome.

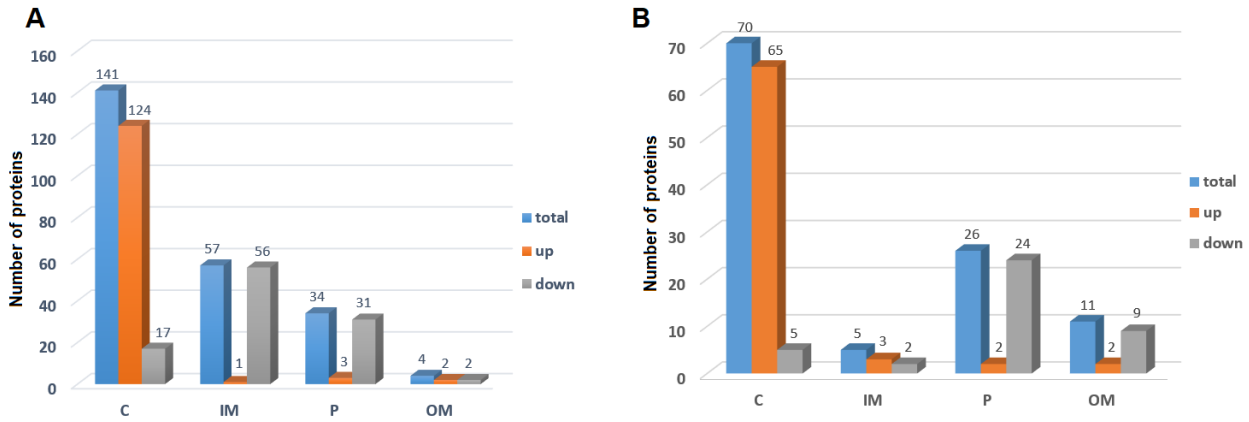

Figure 3.1 Subcellular localisation of quantified proteins according to enrichment or depletion in OMVs. **(A)** Significantly up- and down-regulated proteins in polymyxin B treated polymyxin-susceptible *K. pneumoniae* ATCC700721 OMVs. (B) Significantly up- and down-regulated proteins in polymyxin B treated polymyxin-resistant *K. pneumoniae* ATCC700721 OMVs. The proteins were sorted according to predicted subcellular localisation. C, cytoplasmic; MI, inner membrane; P, periplasm; and OM, outer membrane proteins. Fold-change ≥ 2 and $p < 0.05$ were defined as statistically significant.

3.3.3 Metabolic pathway analysis

Metabolic pathway analysis revealed that 23 pathways were significantly overrepresented in wild type in response to polymyxin B treatment, whereas 12 metabolic pathways were markedly underrepresented (**Tables 3.1 and 3.2**). On the other hand, in the polymyxin-resistant mutant, 20 pathways were overrepresented; and six pathways were under-expressed (**Tables 3.3 and 3.4**). Proteins of carbon metabolism and energy production, pyrimidine metabolism and bacterial secretion pathways were significantly overexpressed in OMVs of sensitive strain in

response to polymyxin B treatment **(Table 3.1)**; whereas those of citric acid cycle, pyrimidine metabolism and Oxidation phosphorylation/ inner membrane electron transport chain were overrepresented in the OMVs of treated resistant strain **(Table 3.3)**. Moreover, polymyxin B treatment of susceptible strain resulted in the production of OMVs richer in lipopolysaccharide and peptidoglycan biosynthesis proteins; however, these proteins were undetected in OMVs of the resistant strain **(Table 3.1, Figures 3.2 and 3.3)**. Interestingly, some Gram-negative strains develop polymyxins resistance either by complete loss of LPS or by its modification (94, 180, 455), leading us to consider the hypothesis that OMVs derived from polymyxin-susceptible *E.coli* would protect the sensitive strain from the action of polymyxin B, while those derived from resistant strain would not confer protection due to the lack of the LPS (456). On the other hand, compared to untreated control, the resistant strain released OMVs rich in host factor-I protein (QS protein), phosphotransferase system enzyme (phosphoenolpyruvate-protein phosphotransferase), RNA degradation as well as nucleotide excision repair and homologous DNA recombination enzymes in response to polymyxin B **(Tables 3.3)**. However, polymyxin B treatment of susceptible strain significantly reduced the amount of a number of QS proteins (Autoinducer 2-binding protein LsrB, high-affinity branched-chain amino acid transport protein, signal peptidase I, zinc metalloprotease and membrane protein insertase YidC) and several of phosphotransferase system enzymes **(Table 3.2)**. Simultaneously, Aminoacyl-tRNAs were also overexpressed in both treated strains, Glycine and threonine-tRNA ligase were upregulated in resistant strain; while in the susceptible strain aspartyl, glycyl, seriyl, Lysyl, proyl and histdyl-tRNA ligase were overrepresented in response to polymyxin B treatment **(Tables 3.1 and 3.2, Figure 3.4)**. It has been found that Aminoacyl-tRNAs play an important role in the biosynthesis of bacterial cell envelope and multiple peptide resistance virulence factors, which control bacterial permeability to cationic antimicrobial compounds via aminoacylation of the inner membrane (457, 458). This finding might explain both the attempt of *K. pneumoniae* to increase the rigidity of its envelope upon exposure to polymyxin B; and the role of OMVs in bacterial resistant to cationic antimicrobial compounds. Similarly, Pyruvate metabolism

enzymes were detected in the OMVs of both treated strains **(Tables 3.1 and 3.3)**, in the susceptible strain, only lactate dehydrogenase was overrepresented in OMVs compared to control untreated strain; whereas, in OMVs of the resistant strain, pyruvate-formate-lyase deactivase and pyruvate-flavodoxin oxidoreductase were overexpressed. Notably, pyruvate-flavodoxin oxidoreductase overexpression has been shown to protect *E.coli* cells from oxidative stress (459), which might explain the over-expression of pyruvate metabolism enzymes in *K. pneumoniae*-derived OMVs following polymyxin B treatment. In addition, the 30S and 50S ribosomal proteins, amino acids biosynthesis enzymes, the sulfate relay system enzyme cysteine-desulferase, glycolysis/gluconeogenesis, UDP-amino sugar, Fatty acid biosynthesis, starch and sucrose metabolic enzymes were upregulated in OMVs of both treated strains compared to control untreated one **(Tables 3.1 and 3.3)**. On contradictory, several proteins of ABC transporters, protein export, two-component system (e.g., sensor protein PhoQ), cationic antimicrobial peptide (CAMP) resistance (notably, 4-amino-4-deoxy-L-arabinose transferase) and beta-lactam resistance (e.g., beta-lactamase extended-spectrum beta-lactamase SHV-11) pathways, were significantly downregulated in OMVs of both strains in response to polymyxin B treatment **(Tables 3.2 and 3.4, Figure 3.5)**. One of polymyxin resistance mechanisms in *K. pneumoniae* involves modification of lipid A with 4-amino-4-deoxy-L-arabinose (432, 434). The two-component regulatory systems [PhoPQ-PmrD]-PmrAB control the expression of 4-amino-4-deoxy-L-arabinose modifications to the lipid A phosphates. These systems are induced by low pH, low magnesium and high iron (432).

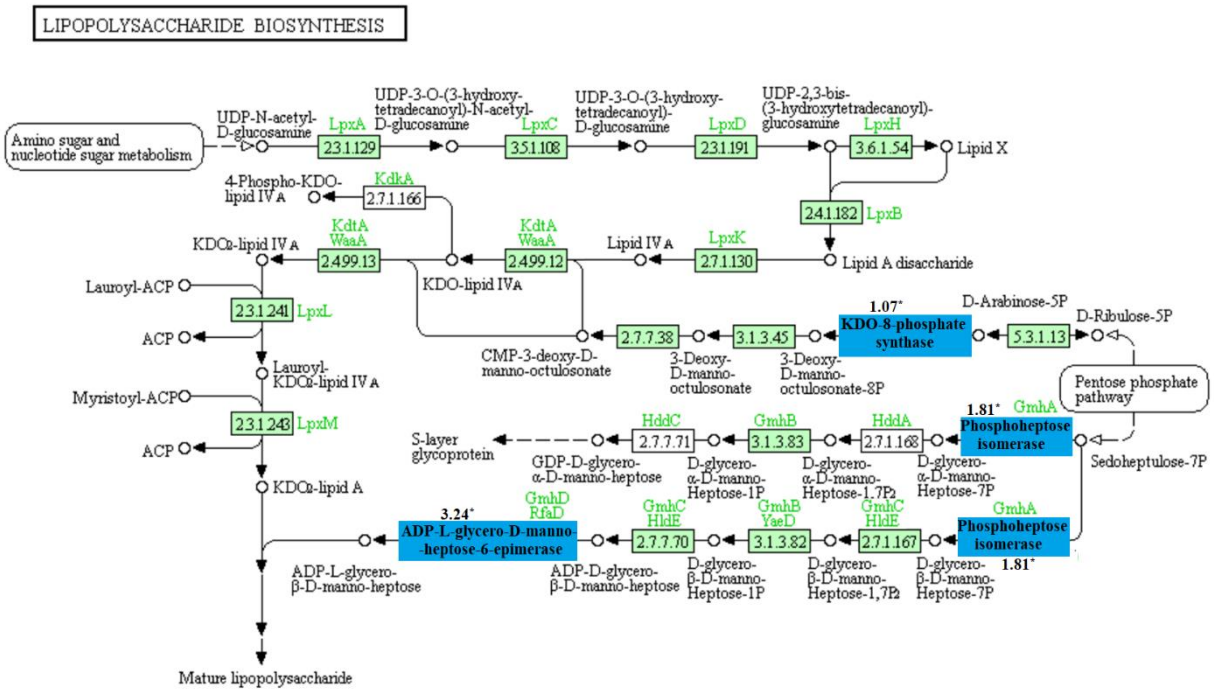

Figure 3.2 Lipopolysaccharide biosynthesis pathway. Enzymes highlighted with (**Blue)** were significantly overexpressed in OMVs of polymyxin B treated polymyxin-susceptible *K. pneumoniae* compared to untreated control. However, no lipopolysaccharide biosynthesis enzymes were detected in OMVs of polymyxin-resistant *K. pneumoniae* in response to polymyxin B treatment. * Fold change of relative intensity of OMVs proteins from polymyxin B-treated polymyxin-susceptible *K. pneumoniae* ATCC 700721 compared to untreated conrol. Statistical significance was calculated with Student's *t* test. $P<0.05$ was defined as statistically significant.

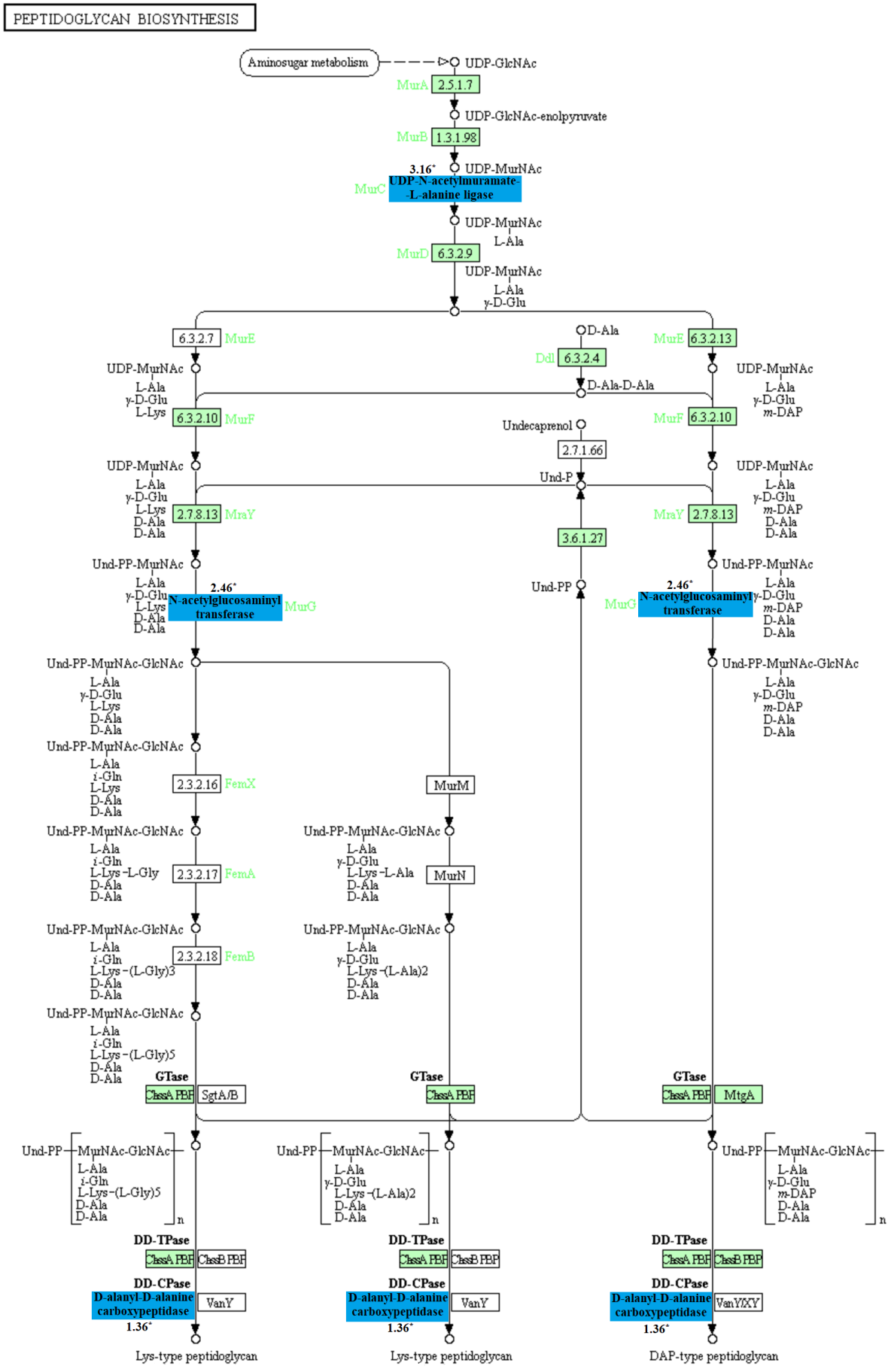

Figure 3.3 Peptidoglycan biosynthesis pathway. Enzymes highlighted with **(Blue)** were significantly upregulated in OMVs of polymyxin B treated polymyxin-susceptible *K. pneumoniae* compared to untreated control. However, no peptidoglycan biosynthesis enzymes were detected in OMVs of

polymyxin-resistant *K. pneumoniae* in response to polymyxin B treatment. * Fold change of relative intensity of OMVs proteins from polymyxin B-treated polymyxin-susceptible *K. pneumoniae* ATCC 700721 compared to untreated conrol. Statistical significance was calculated with Student's *t* test. P<0.05 was defined as statistically significant.

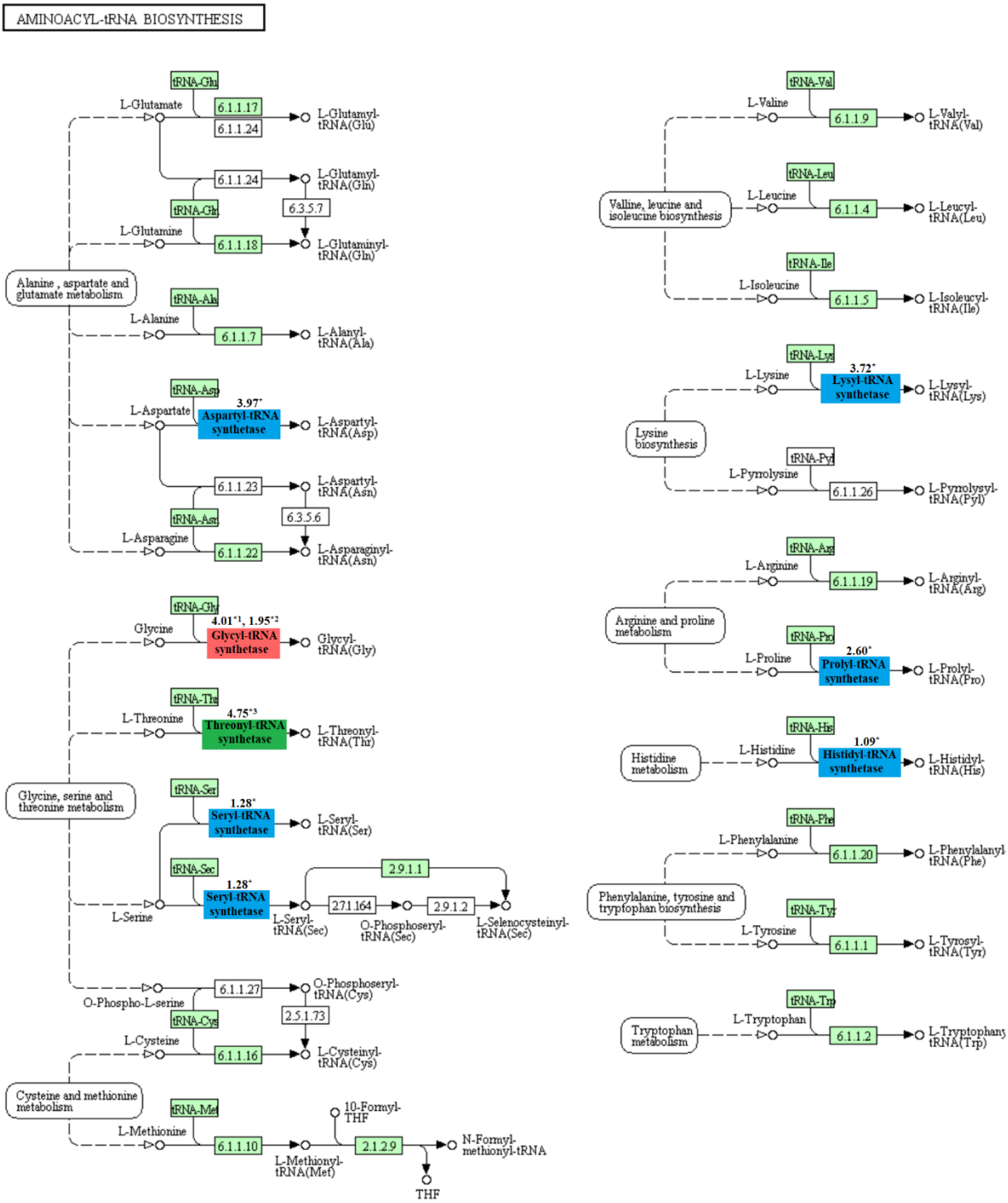

Figure 3.4 Aminoacyl-tRNA biosynthesis pathway. Enzymes highlighted with (**Blue)** were significantly overexpressed in OMVs of polymyxin B treated polymyxin-susceptible *K. pneumoniae* compared to untreated control. While the enzyme highlighted with (**green)** was upregulated in OMVs of polymyxin-resistant *K. pneumoniae* in response to polymyxin B. The enzyme highlighted with (**red)**

was overrepresented in OMVs of both strains following polymyxin B treatment compared to control untreated strains. * Fold change of relative intensity of OMVs proteins from both polymyxin B-treated polymyxin-susceptible and -resistant *K. pneumoniae* ATCC 700721 compared to untreated conrol. Statistical significance was calculated with Student's *t* test. P<0.05 was defined as statistically significant. [1] fold change of relative intensity of OMVs Glycyl-tRNA synthetase alpha subunit from polymyxin B-treated polymyxin-susceptible *K. pneumoniae* ATCC 700721 compared to untreated control. [2] fold change of relative intensity of OMVs Glycyl-tRNA synthetase alpha subunit from polymyxin B-treated polymyxin-resistant *K. pneumoniae* ATCC 700721 compared to untreated control. [3] fold change of relative intensity of OMVs Threonyl-tRNA synthetase from polymyxin B-treated polymyxin-resistant *K. pneumoniae* ATCC 700721 compared to untreated control.

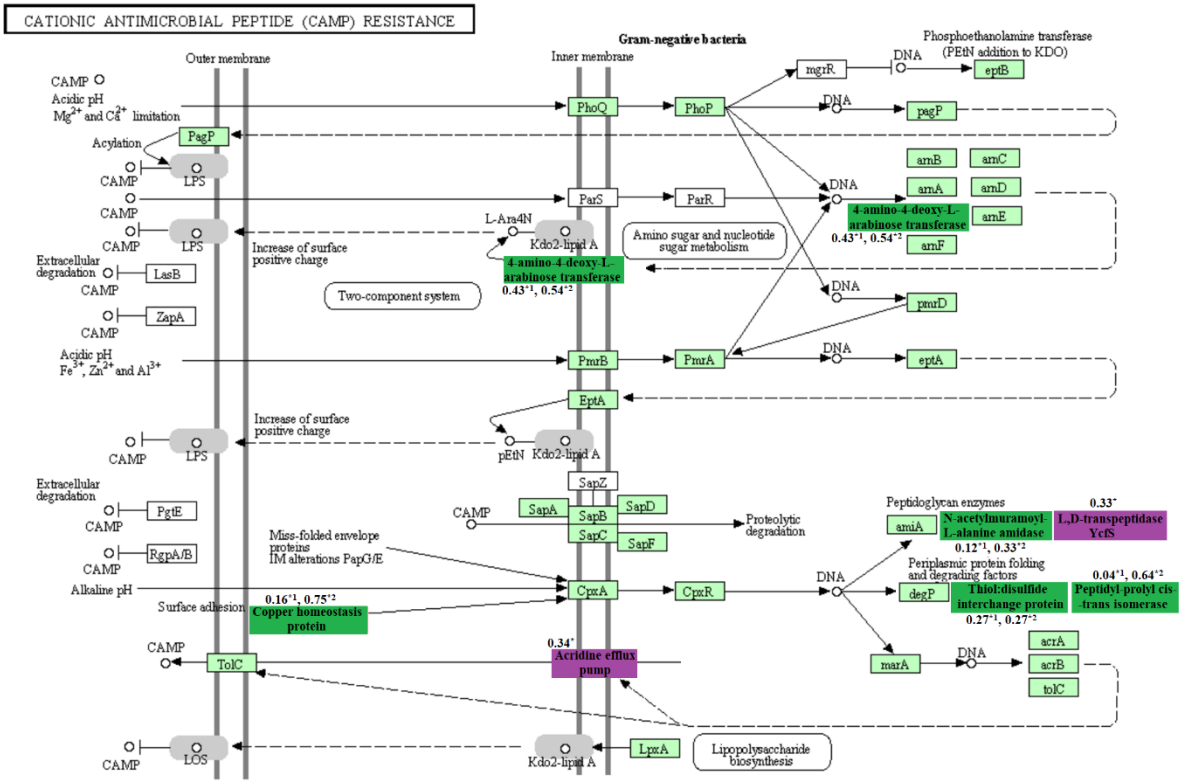

Figure 3.5 Cationic antimicrobial peptide (CAMP) resistance pathway. The enzyme highlighted with **(Purple)** was significantly downregulated in OMVs of polymyxin B treated polymyxin-susceptible *K. pneumoniae* compared to untreated control. While, enzymes highlighted with **(Green)** were underrepresented in OMVs of both strains following polymyxin B treatment compared to control untreated strains. * Fold change of relative intensity of OMVs proteins from both polymyxin B-treated polymyxin-susceptible and -resistant *K. pneumoniae* ATCC 700721 compared to untreated conrol. Statistical significance was calculated with Student's t test. P<0.05 was defined as statistically significant. [1] fold change of relative intensity of OMVs proteins from polymyxin B-treated polymyxin-susceptible *K. pneumoniae* ATCC 700721 compared to untreated control. [2] fold change of relative intensity of OMVs proteins from polymyxin B-treated polymyxin-resistant *K. pneumoniae* ATCC 700721 compared to untreated control.

3.4 Conclusions

In this study, we show that polymyxin B treatment of both susceptible and resistant *K. pneumoniae* strains significantly reduced the cytoplasmic, inner membrane and periplasmic protein content of their OMVs, compare to the untreated control. In addition, our proteomics analysis reveals that OMVs proteins from several bacterial biological processes, notably lipopolysaccharide and peptidoglycan biosynthesis pathways were overrepresented in the OMVs proteome of the polymyxin B treated susceptible stain; whereas; in the resistant strain, QS, phosphotransferase system, RNA degradation and nucleotide excision repair pathways were significantly overrepresented. It is important to highlight that the lipid A in the Gram-negative OM is the primary target for polymyxins; therefore, these antibiotics exhibit a narrow spectrum of activity against Gram-negative bacteria that do not express LPS. These OMVs proteomic changes may be accompanied by the remodelling of the bacterial OM, in particular, the modification of lipid A, which together serve to make the *K. pneumoniae* OM and OMVs invincible to polymyxin attack.

Table 3.1 Metabolic pathway categorization of statistically significantly overrepresented proteins in OMVs from polymyxin B-treated polymyxin-susceptible *K. pneumoniae* ATCC 700721 compared to untreated control.

Metabolic pathway	**Protein name**	**Fold K.pS treated/K.pS control change***
Ribosome		
	30S ribosomal protein S10	4.71
	50S ribosomal protein L4	4.46
	50S ribosomal protein L23	3.85
	50S ribosomal protein L16	2.27
	30S ribosomal protein S8	3.5
	50S ribosomal protein L6	2.75
	50S ribosomal protein L18	2.43
	30S ribosomal protein S5	2.39
	30S ribosomal protein S4	3.58
	50S ribosomal protein L17	2.25
	50S ribosomal protein L13	2.19
	30S ribosomal protein S9	1.6
	30S ribosomal protein S7	2.12
	50S ribosomal protein L10	2.1
	50S ribosomal protein L1	1.73

	50S ribosomal protein L9	1.59
	30S ribosomal protein S18	2.04
	50S ribosomal protein L21	2.41
	30S ribosomal protein S20	1.74
	30S ribosomal protein S21	2.08
	50S ribosomal protein L2	1.72
	30S ribosomal protein S19	1.66
	50S ribosomal protein L22	2.17
	30S ribosomal protein S3	1.54
	50S ribosomal protein L29	1.58
	30S ribosomal protein S17	2.22
	50S ribosomal protein L14	1.57
	50S ribosomal protein L24	1.55
	50S ribosomal protein L5	1.5
	50S ribosomal protein L30	1.44
	50S ribosomal protein L15	1.39
	30S ribosomal protein S13	1.35
	30S ribosomal protein S11	1.34
	50S ribosomal protein L7/L12	1.33

	50S ribosomal protein L11	1.3
	30S ribosomal protein S2	1.27
	30S ribosomal protein S15	1.24
	30S ribosomal protein S6	1.13
	50S ribosomal protein L28	1.22
	50S ribosomal protein L27	1.6
	30S ribosomal protein S16	1.76
	50S ribosomal protein L19	2.92
	50S ribosomal protein L25	2.81
Biosynthesis of amino acids (including Shikimate pathway)		
	Ribose-phosphate pyrophosphokinase (RPPK)	5.7
	Imidazole glycerol phosphate synthase subunit HisF	3.11
	6-phosphofructokinase	4.13
	Phosphoglycerate kinase	2.45
	3-dehydroquinate dehydratase	2.09
	Probable phosphoglycerate mutase GpmB	1.97
	Phosphoserine aminotransferase	2.34
	Enolase	1.62

	Pyruvate kinase	1.65
	Acetolactate synthase small subunit	1.75
	S-adenosylmethionine synthase (AdoMet synthase)	3.68
	Gamma-glutamyl phosphate reductase (GPR)	1.83
Carbon metabolism		
	Glucose-6-phosphate 1-dehydrogenase (G6PD)	1.41
	Glucose-6-phosphate isomerase (GPI)	1.23
	Fructose-1,6-bisphosphatase class 1 (FBPase class 1)	1.31
	Pyruvate dehydrogenase E1 component	2.32
	Phosphate acetyltransferase	2.68
	Acetate kinase	3.74
	Dihydrolipoyl dehydrogenase	1.04
	succinyl-CoA synthetase subunit beta	1.08
	Probable malate:quinone oxidoreductase	1.12
Purine metabolism		
	Phosphoglucomutase	5.07
	Phosphoribosylaminoimidazole-succinocarboxamide synthase	1.52
	Adenine phosphoribosyltransferase (APRT)	2.69
	Nucleoside diphosphate kinase (NDK) (NDP kinase)	1.51

	Adenylosuccinate synthetase (AMPSase)	4.97
	DNA-directed RNA polymerase subunit alpha	3.92
	Guanylate kinase	2.8
	Ribonucleotide-diphosphate reductase beta subunit	3.94
	Hypoxanthine-guanine phosphoribosyltransferase	1.2
	Adenosine deaminase	2.67
Glycolysis / Gluconeogenesis		
	triosephosphate isomerase	4.38
	Glyceraldehyde-3-phosphate dehydrogenase	1.53
	Acetyltransferase component of pyruvate dehydrogenase complex	3.87
Pyrimidine metabolism		
	Aspartate carbamoyltransferase (EC 2.1.3.2) (Aspartate transcarbamylase) (ATCase)	1.56
	Orotate phosphoribosyltransferase	2.99
	Orotidine 5'-phosphate decarboxylase	3.28
	DNA-directed RNA polymerase subunit beta	1.69
	Uridylate kinase (UK)	3.12
	Uracil phosphoribosyltransferase	2.57
Starch and sucrose metabolism		
	Fructokinase	1.11

	UTP--glucose-1-phosphate uridylyltransferase	1.03
	Glucose-1-phosphate adenylyltransferase	2.89
	4-alpha-glucanotransferase	2.56
	Pullulanase protein	3.43
	Putative PEP-dependent phosphotransferase enzyme III	1.14
Amino sugar and nucleotide sugar metabolism		
	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	1.29
	D-fructose-6-phosphate amidotransferase	4.26
	UDP-glucose dehydrogenase	1.19
	UDP-galactose 4-epimerase	2.54
Pentose phosphate pathway		
	Ribose-5-phosphate isomerase A	2.15
Glycine, serine and threonine metabolism		1.15
	2-amino-3-ketobutyrate coenzyme A ligase (AKB ligase)	2.65
	L-threonine 3-dehydrogenase (TDH)	4.37
	8-amino-7-oxononanoate synthase	1.25
Aminoacyl-tRNA biosynthesis		
	Aspartyl-tRNA synthetase (AspRS)	3.97

	Glycyl-tRNA synthetase alpha subunit (GlyRS)	4.01
	Seryl-tRNA synthetase (SerRS)	1.28
	Lysyl-tRNA synthetase (LysRS)	3.72
	Prolyl-tRNA synthetase (ProRS)	2.6
	Histidyl-tRNA synthetase (HisRS)	1.09
Cysteine and methionine metabolism		
	L-serine deaminase 2	1.02
	Aminotransferase	3.61
Alanine, aspartate and glutamate metabolism		
	Asparagine synthetase B	1.05
Fatty acid biosynthesis		
	3-oxoacyl-(Acyl carrier protein) synthase	1.45
	3-ketoacyl-(Acyl-carrier-protein) reductase	1.26
	3-hydroxyacyl-[acyl-carrier-protein] dehydratase FabZ	2.23
	Enoyl-[acyl-carrier-protein] reductase [NADH]	3.46
Lipopolysaccharide biosynthesis		
	2-dehydro-3-deoxyphosphooctonate aldolase (KDO-8-phosphate synthase)	1.07
	Phosphoheptose isomerase	1.81

	ADP-L-glycero-D-manno-heptose-6-epimerase	3.24
Peptidoglycan biosynthesis		
	UDP-N-acetylglucosamine--N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase	2.46
	D-alanyl-D-alanine carboxypeptidase, fraction A penicillin-binding protein 5	1.36*
	UDP-N-acetylmuramate--L-alanine ligase	3.16
Phenylalanine metabolism		
	Acetyl-CoA acetyltransferase	3
Glutathione metabolism		
	Aminoacyl-histidine dipeptidase (Peptidase D)	1.55
	Glutathione synthetase	1.21
Bacterial secretion system		
	Protein-export protein SecB	2.7
	Signal recognition particle receptor FtsY (SRP receptor)	3.2
Base excision repair		
	Exonuclease III	2.91
Sulfur relay system		
	Cysteine desulfurase IscS	1.79

One carbon pool by folate		
	Bifunctional protein FolD [Includes: Methylenetetrahydrofolate dehydrogenase; Methenyltetrahydrofolate cyclohydrolase]	2.55
Lipoic acid metabolism		
	Lipoyl synthase	1.32
Pyruvate metabolism		
	L-lactate dehydrogenase (EC 1.1.-.-)	1.71

* Fold change of relative intensity of OMVs proteins from polymyxin B-treated polymyxin-susceptible *K. pneumoniae* ATCC 700721 compared to untreated conrol. Statistical significance was calculated with Student's *t* test. $P<0.05$ was defined as statistically significant.

Table 3.2 Metabolic pathway categorization of statistically significantly underrepresented proteins in OMVs from polymyxin B-treated polymyxin-susceptible *K. pneumoniae* ATCC 700721 compared to untreated control.

Metabolic pathway	**Protein name**	**Fold K.p S treated/K.p S control change***
ABC transporters		
	Molybdate transport protein (ABC superfamily, peri_bind)	0.75
	Putative ABC transporter periplasmic binding protein	0.34
	Thiamin-binding periplasmic protein	0.29
	Glycine/betaine/proline transport protein (ABC superfamily, peri_bind)	0.82
	Putative glycine/betaine/choline transport protein, osmoprotection (ABC superfamily, peri_bind)	0.07

	Periplasmic maltose-binding protein	0.58
	Maltose transport protein (ABC superfamily, membrane)	0.23
	Maltose/maltodextrin import ATP-binding protein MalK	0.1
	Putative transport protein (ABC superfamily, atp_bind and membrane)	0.09
	Putative transport protein (ABC superfamily, peri_bind)	0.3
	Uncharacterized protein	0.21
	L-arabinose-binding periplasmic protein (ABP)	0.32
	Galactose transport protein (ABC superfamily, peri_bind)	0.4
	Putative periplasmic binding protein	0.97
	sn-glycerol 3-phosphate transport protein (ABC superfamily, peri_bind)	0.01
	High-affinity zinc uptake system periplasmic protein	0.46
	Putative iron-regulated membrane protein	0.04
	Glutathione transport system substrate-binding protein	0.15
	Cationic peptide transport system substrate-binding protein	0.16
	Cationic peptide transport system ATP-binding protein	0.02
	Dipeptide transport protein	0.57
	Putative bacterial extracellular solute-binding protein	0.12
	Oligopeptide transporter ATP-binding component	0.2
	D-methionine transport protein (ABC superfamily, peri_bind)	0.17

	Methionine import ATP-binding protein MetN	0.08
	Cysteine transport protein (ABC superfamily, peri_bind)	0.23
	Putative ATP-binding component of a transport system	0.06
	Arginine 3rd transport system periplasmic binding protein	0.48
	ATP-binding component of 3rd arginine transport system	0.41
	Glutamine ABC transporter periplasmic-binding protein	0.55
	Histidine transport protein (ABC superfamily, peri_bind)	0.31
	Phosphate import ATP-binding protein PstB (EC 3.6.3.27) (ABC phosphate transporter) (Phosphate-transporting ATPase)	0.43
	O-antigen export-NBD component	0.54
	Lipid A export ATP-binding/permease protein MsbA (EC 3.6.3.-)	0.66
	Lipopolysaccharide export system permease protein	0.56
	ATP-binding component of cytochrome-related transport	0.53
	Cell division membrane protein	0.13
	ATP-binding component of a membrane-associated complex involved in cell division	0.38
Oxidative phosphorylation		
	Polyphosphate kinase (EC 2.7.4.1) (ATP-polyphosphate phosphotransferase) (Polyphosphoric acid kinase)	0.05
	NADH-quinone oxidoreductase subunit A (EC 1.6.5.11) (NADH dehydrogenase I subunit A) (NDH-1 subunit A) (NUO1)	0.18
	NADH-quinone oxidoreductase subunit B (EC 1.6.5.11) (NADH dehydrogenase I subunit B) (NDH-1 subunit B)	0.19

	NADH-quinone oxidoreductase subunit C/D (EC 1.6.5.11) (NADH dehydrogenase I subunit C/D) (NDH-1 subunit C/D)	0.61
	ATP synthase subunit E	0.03
	NADH dehydrogenase I chain F	0.52
	NADH-quinone oxidoreductase (EC 1.6.5.11)	0.35
	NADH-quinone oxidoreductase subunit I (EC 1.6.5.11) (NADH dehydrogenase I subunit I) (NDH-1 subunit I)	0.51
	ATP synthase subunit alpha (EC 3.6.3.14) (ATP synthase F1 sector subunit alpha) (F-ATPase subunit alpha)	0.49
	ATP synthase subunit beta (EC 3.6.3.14) (ATP synthase F1 sector subunit beta) (F-ATPase subunit beta)	0.25
	ATP synthase subunit b (ATP synthase F(0) sector subunit b) (ATPase subunit I) (F-type ATPase subunit b) (F-ATPase subunit b)	0.44
	ATP synthase subunit c (ATP synthase F(0) sector subunit c) (F-type ATPase subunit c) (F-ATPase subunit c) (Lipid-binding protein)	0.5
	Succinate dehydrogenase hydrophobic membrane anchor subunit	0.33
	Succinate dehydrogenase flavoprotein subunit (EC 1.3.5.1)	0.37
	Cytochrome o ubiquinol oxidase subunit I	0.92
	Ubiquinol oxidase subunit 2	0.47
Two-component system		
	Sensor protein PhoQ	0.04
	Phosphate-binding protein PstS	0.36
	Acid phosphatase (EC 3.1.3.2)	0.27
	Two-component system, OmpR family, sensor histidine kinase CpxA	0.45

	Multidrug resistance protein MdtA (Multidrug transporter MdtA)	0.39
	Aerobic respiration control sensor protein (EC 2.7.13.3)	0.28
	Cryptic nitrate reductase 2 alpha subunit	0.14
	Fumarate reductase flavoprotein subunit (EC 1.3.5.4)	0.87
	Succinate dehydrogenase iron-sulfur subunit (EC 1.3.5.1)	0.76
	Outer membrane lipoprotein RcsF	0.11
	Cryptic nitrate reductase 2 beta subunit	0.94
	Zn-binding periplasmic protein	0.74
	ATP-binding protein of glutamate/aspartate transport system	0.72
	Glutamate/aspartate transport protein (ABC superfamily, membrane)	0.78
	Glutamate/aspartate periplasmic binding protein	0.67
	Cytochrome oxidase bd-II, subunit I	0.18
Quorum sensing		
	Autoinducer 2-binding protein LsrB (AI-2-binding protein LsrB)	0.62
	High-affinity branched-chain amino acid transport protein (ABC superfamily, peri_bind)	0.45
	Signal peptidase I (EC 3.4.21.89)	0.71
	Zinc metalloprotease (EC 3.4.24.-)	0.62
	Membrane protein insertase YidC (Foldase YidC) (Membrane integrase YidC) (Membrane protein YidC)	0.71
	Protein translocase subunit SecA	0.52

Cationic antimicrobial peptide (CAMP) resistance		
	4-amino-4-deoxy-L-arabinose lipid A transferase (lipid A modification)	0.43
	N-acetylmuramoyl-L-alanine amidase	0.12
	L,D-transpeptidase YcfS	0.33
	Copper homeostasis protein	0.16
	Acridine efflux pump	0.34
	Thiol:disulfide interchange protein	0.27
	Peptidyl-prolyl cis-trans isomerase (PPIase) (EC 5.2.1.8)	0.04
Beta-Lactam resistance		
	Bifunctional penicillin-binding protein 1a: transglycosylase/transpeptidase	0.13
	Beta-lactamase (EC 3.5.2.6)	0.16
Phosphotransferase system (PTS)		
	PTS family enzyme IIC/enzyme IIB	0.29
	PTS family enzyme IIC/enzyme IIB/enzyme IIC	0.26
	PTS family enzyme IICB/enzyme IIA	0.46
	PTS enzyme IID, mannose-specific	0.25
	PTS enzyme IIAB, mannose-specific	0.28

	PTS family enzyme IIA/FPr	0.46
	PTS family enzyme IIB'BC, fructose-specific	0.49
Protein export		
	Protein translocase subunit SecD	0.49
	Preprotein translocase subunit YajC	0.52
	Sec-independent protein translocase protein TatA	0.27
Glycerophospholipid metabolism		
	Glycerol-3-phosphate acyltransferase (GPAT) (EC 2.3.1.15)	0.26
	Glycerophosphodiester phosphodiesterase, periplasmic	0.22
	CDP-diacylglycerol pyrophosphatase (EC 3.6.1.26) (CDP-diacylglycerol phosphatidylhydrolase) (CDP-diglyceride hydrolase)	0.38
	CDP-diacylglycerol--serine O-phosphatidyltransferase (EC 2.7.8.8)	0.14
	Non-essential phosphatidylglycerophosphate phosphatase, membrane bound	0.29
RNA degradation		
	Ribonuclease E (RNase E) (EC 3.1.26.12)	0.93
	ATP-dependent RNA helicase RhlB (EC 3.6.4.13)	0.03
	ATP-dependent RNA helicase DeaD (EC 3.6.4.13) (Cold-shock DEAD box protein A)	0.64
	Poly(A) polymerase I (PAP I) (EC 2.7.7.19)	0.17
	Tetraacyldisaccharide 4'-kinase (EC 2.7.1.130) (Lipid A 4'-kinase)	0.77

Nucleotide excision repair		
	UvrABC system protein A (UvrA protein) (Excinuclease ABC subunit A)	0.35

* Fold change of relative intensity of OMVs proteins from polymyxin B-treated polymyxin-susceptible *K. pneumoniae* ATCC 700721 compared to untreated conrol. Statistical significance was calculated with Student's *t* test. P<0.05 was defined as statistically significant.

Table 3.3 Metabolic pathway categorization of statistically significantly overrepresented proteins in OMVs from polymyxin B-treated polymyxin-resistant *K. pneumoniae* ATCC 700721 compared to untreated control.

Metabolic pathway	**Protein name**	**Fold K.pR treated/K.pR control change***
Ribosome		
	30S ribosomal protein S10	1.72
	50S ribosomal protein L3	2.05
	50S ribosomal protein L4	1.97
	50S ribosomal protein L23	2.91
	50S ribosomal protein L16	2.54
	30S ribosomal protein S8	1.94
	50S ribosomal protein L6	2.39
	50S ribosomal protein L18	2.78
	30S ribosomal protein S5	2.07
	30S ribosomal protein S4	2.18

	50S ribosomal protein L17	2.68
	50S ribosomal protein L13	1.69
	30S ribosomal protein S9	2.43
	30S ribosomal protein S7	2.37
	50S ribosomal protein L10	2.57
	50S ribosomal protein L1	1.99
	50S ribosomal protein L9	6.32
	30S ribosomal protein S18	3.17
	50S ribosomal protein L21	2.69
	30S ribosomal protein S20	3.3
	30S ribosomal protein S21	3.89
Biosynthesis of amino acids (including Shikimate pathway)		2.6
	Phosphofructokinase	5.26
	3-dehydroquinate synthase	3.47
	3-phosphoshikimate 1-carboxyvinyltransferase	7.07
	Enolase (EC 4.2.1.11) (2-phospho-D-glycerate hydro-lyase) (2-phosphoglycerate dehydratase)	2.01
	L-serine deaminase 2	7.36
	Serine hydroxymethyltransferase	6.86
	Argininosuccinate synthase	2.85

Glycolysis / Gluconeogenesis		
	6-phosphofructokinase II	5.3
	triosephosphate isomerase	7.24
	Glyceraldehyde-3-phosphate dehydrogenase	3.34
	Pyruvate dehydrogenase E1 component	5.68
	Aldehyde-alcohol dehydrogenase	5.92
RNA degradation		
	Ribonuclease E (RNase E)	7.51
	ATP-dependent RNA helicase RhlB	2.99
	chaperonin GroEL	3.27
	RNA-binding protein Hfq	6.31
	Polyphosphate kinase	3.21
Citrate cycle (TCA cycle)		
	Succinyl-CoA synthetase subunit beta	3.15
	2-oxoglutarate decarboxylase	1.76
Oxidative phosphorylation		
	NADH-quinone oxidoreductase subunit I	2.63
	ATP synthase subunit delta	4.86
	NADH dehydrogenase gamma subunit	3.12

Pyrimidine metabolism		
	Orotate phosphoribosyltransferase	3.37
	Carbamoyl-phosphate synthase large chain	2.61
	Uracil phosphoribosyltransferase	1.86
	DNA polymerase III subunit beta	2.02
Alanine, aspartate and glutamate metabolism		
	Adenylosuccinate synthetase	2.06
	Carbamoyl-phosphate synthase small subunit	4.84
Starch and sucrose metabolism		
	Trehalose-6-phosphate synthase (TPS)	2.4
	UTP--glucose-1-phosphate uridylyltransferase	2.11
	Alpha-1,4 glucan phosphorylase	1.64
Amino sugar and nucleotide sugar metabolism		
	UDP-glucose dehydrogenase	1.79
	UDP-glucose pyrophosphorylase	1.92
	UDP-galactose 4-epimerase	2.08
Glycine, serine and threonine metabolism		
	L-serine dehydratase	5.7

	Glycine hydroxymethyltransferase	2.47
	Oxygen-dependent choline dehydrogenase	1.55
Homologous recombination		
	DNA polymerase III sub-unit alpha	2.3
	Holliday junction ATP-dependent DNA helicase RuvB	3.28
Nucleotide excision repair		
	UvrABC system protein B (Protein UvrB) (Excinuclease ABC subunit B)	2.18
	DNA helicase	2.48
	dTDP-4-dehydrorhamnose reductase	3.16
Aminoacyl-tRNA biosynthesis		
	Glycine--tRNA ligase beta subunit	1.95
	Threonyl-tRNA synthetase	4.75
Fatty acid biosynthesis		
	3-oxoacyl-(Acyl carrier protein) synthase	1.94
	3-ketoacyl-(Acyl-carrier-protein) reductase	2.26
Pentose phosphate pathway		
	Ribose-5-phosphate isomerase A	2.17
Quorum sensing		
	host factor-I protein	1.96

	Signal recognition particle receptor FtsY (SRP receptor)	3.46
Pyruvate metabolism		
	Pyruvate-formate-lyase deactivase	1.89
	Pyruvate-flavodoxin oxidoreductase	2.27
Phosphotransferase system (PTS)		
	Phosphoenolpyruvate-protein phosphotransferase (Phosphotransferase system, enzyme I)	2.88
Sulfur relay system		
	2-dehydro-3-deoxyphosphooctonate aldolase	3.82
	Cysteine desulfurase IscS	4.09

* Fold change of relative intensity of OMVs proteins from polymyxin B-treated polymyxin-resistant *K. pneumoniae* ATCC 700721 compared to untreated control. Statistical significance was calculated with Student's *t* test. $P<0.05$ was defined as statistically significant.

Table 3.4 Metabolic pathway categorization of statistically significantly underrepresented proteins in OMVs from polymyxin B-treated polymyxin-resistant *K. pneumoniae* ATCC 700721 compared to untreated control.

Metabolic pathway	**Protein name**	**Fold K.pR treated/K.pR control change***
ABC transporters		
	Sulfate transport system substrate-binding protein	0.72
	Iron(III) transport system substrate-binding protein	0.73
	Putrescine-binding periplasmic protein	0.53

	Glycine/betaine/proline transport protein (ABC superfamily, peri_bind)	0.62
	Osmoprotectant transport system substrate-binding protein	0.11
	Periplasmic maltose-binding protein	0.92
	Oligogalacturonide transport system substrate-binding protein	0.97
	Phospholipid transport system substrate-binding protein	0.71
	D-ribose periplasmic binding protein	0.65
	Galactose transport protein (ABC superfamily, peri_bind)	0.19
	D-xylose transport protein (ABC superfamily, peri_bind)	0.77
	Inositol transport system substrate-binding protein	0.68
	sn-glycerol 3-phosphate transport protein (ABC superfamily, peri_bind)	0.66
	Phosphate-binding protein PstS	0.12
	Lysine-, arginine-, ornithine-binding periplasmic protein	0.06
	Histidine transport protein (ABC superfamily, peri_bind)	0.15
	Glutamine ABC transporter periplasmic-binding protein	0.74
	Arginine 3rd transport system periplasmic binding protein	0.58

	Glutamate/aspartate periplasmic binding protein	0.1
	Cysteine transport protein (ABC superfamily, peri_bind)	0.45
	High-affinity branched-chain amino acid transport protein (ABC superfamily, peri_bind)	0.43
	D-methionine transport protein (ABC superfamily, peri_bind)	0.55
	Putative bacterial extracellular solute-binding protein	0.42
	Dipeptide transport protein	0.24
	Putative dipeptide transport protein (ABC superfamily, peri_bind)	0.49
	Iron complex transport system substrate-binding protein	0.16
Beta-Lactam resistance		
	extended-spectrum beta-lactamase SHV-11	0.5
Two-component system		
	Alkaline phosphatase	0.08
	outer membrane protein, Cu(I)/Ag(I) efflux system	0.07
Cationic antimicrobial peptide (CAMP) resistance		
	4-amino-4-deoxy-L-arabinose lipid A transferase (lipid A modification)	0.54
	Copper homeostasis protein	0.75

	N-acetylmuramoyl-L-alanine amidase	0.33
	Thiol:disulfide interchange protein DsbA	0.27
	Peptidyl-prolyl cis-trans isomerase (PPIase)	0.64
Purine metabolism		
	UDP-sugar hydrolase (5'-nucleotidase)	0.26
	2',3'-cyclic nucleotide 2'-phosphodiesterase/3'-nucleotidase bifunctional periplasmic protein	0.34
Protein export		
	Sec-independent protein translocase protein TatA	0.51

[*] Fold change of relative intensity of OMVs proteins from polymyxin B-treated polymyxin-resistant *K. pneumoniae* ATCC 700721 compared to untreated conrol. Statistical significance was calculated with Student's *t* test. $P<0.05$ was defined as statistically significant.

Chapter 4: Lipidomic composition of outer membrane vesicles from paired polymyxin-susceptible and -resistant *Klebsiella pneumoniae* clinical isolates

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Lipidomic Analysis of the Outer Membrane Vesicles from Paired Polymyxin-Susceptible and -Resistant *Klebsiella pneumoniae* Clinical Isolates

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Abstract: Gram-negative bacteria produce outer membrane vesicles (OMVs) as delivery vehicles for nefarious bacterial cargo such as virulence factors, which are antibiotic resistance determinants. This study aimed to investigate the impact of polymyxin B treatment on the OMV lipidome from paired polymyxin-susceptible and -resistant *Klebsiella pneumoniae* isolates. *K. pneumoniae* ATCC 700721 was employed as a reference strain in addition to two clinical strains, *K. pneumoniae* FADDI-KP069 and *K. pneumoniae* BM3. Polymyxin B treatment of the polymyxin-susceptible strains resulted in a marked reduction in the glycerophospholipid, fatty acid, lysoglycerophosphate and sphingolipid content of their OMVs. Conversely, the polymyxin-resistant strains expressed OMVs richer in all of these lipid species, both intrinsically and increasingly under polymyxin treatment. The average diameter of the OMVs derived from the *K. pneumoniae* ATCC 700721 polymyxin-susceptible isolate, measured by dynamic light scattering measurements, was ~90.6 nm, whereas the average diameter of the OMVs isolated from the paired polymyxin-resistant isolate was ~141 nm. Polymyxin B treatment (2 mg/L) of the *K. pneumoniae* ATCC 700721 cells resulted in the production of OMVs with a larger average particle size in both the susceptible (average diameter ~124 nm) and resistant (average diameter
~154 nm) strains. In light of the above, we hypothesize that outer membrane remodelling associated with polymyxin resistance in *K. pneumoniae* may involve fortifying the membrane structure with increased glycerophospholipids, fatty acids, lysoglycerophosphates and sphingolipids. Putatively, these changes serve to make the outer membrane and OMVs more impervious to polymyxin attack.

Keywords: outer membrane vesicles; lipidomics; Gram-negative; polymyxin; extremely drug resistant

4.1 Introduction

Over the last decade, extremely-drug resistant (XDR) *K. pneumoniae* has emerged as one of the most deadly Gram-negative 'superbugs' (413-415). *K. pneumoniae* is responsible for numerous lethal nosocomial outbreaks (416); more worryingly, the mortality of nosocomial *K. pneumoniae* infections can be up to 50% (417). Carbapenem resistance in *K. pneumoniae* mediated by carbapenemase was firstly reported in 1996 in the New York City and has spread to most global centres (417, 418). In 2008, bla_{NDM-1} which encodes the class B NDM-1 that inactivates carbapenems, was first detected in a Swedish patient of who had contracted an infection in India (419). Polymyxins (i.e., colistin and polymyxin B) are increasingly used as the last-line therapy against XDR *K. pneumoniae* (15). Indeed, considerable *in vitro* activity against *K. pneumoniae* strains has been demonstrated (136); 98.2 % of general clinical strains of *K. pneumoniae* are susceptible to polymyxin B and colistin (420-425). Ominously, XDR strains that are resistant to polymyxins have recently emerged (426, 427), which highlights the need for a greater appreciation of the mechanism(s) of polymyxin resistance in *K. pneumoniae* to assist targeted drug discovery strategies.

The Gram-negative OM constitutes a formidable barrier limiting the permeability of various noxious substances such as antimicrobial drugs (156, 428). This complex asymmetrical structure comprises an inner phospholipid leaflet, as well as an outer leaflet that predominantly contains LPS, proteins and phospholipids. Additionally, *K. pneumoniae* commonly expresses a capsular polysaccharide that coats the OM, the expression levels of which have been related to polymyxin susceptibility (429-432). The antimicrobial action of polymyxins is mediated through a direct and very specific interaction with the lipid A component of the LPS which, leads to a disruption of the OM barrier (15). The cationic L-α,γ-Dab residues of the polymyxin molecule produce an electrostatic attraction to the negatively charged lipid A phosphate groups, displacing the divalent cations (Mg^{2+} and Ca^{2+}) (15). The displacement leads to the disorganization of the LPS leaflet, enabling the insertion of the hydrophobic tail and the hydrophobic side chains of amino acids 6 and 7 of the polymyxin molecule into the OM (433).

Polymyxin resistance in *K. pneumoniae* primarily involves the multi-tier up-regulation of capsular polysaccharide expression, and the systems required for the modification of lipid A with 4-amino-4-deoxy-L-arabinose and palmitoyl addition (173, 429, 432, 434-440). In *K. pneumoniae* the expression of 4-amino-4-deoxy-L-arabinose modifications to the lipid A phosphates is under control of the two component regulatory systems [PhoPQ-PmrD]-PmrAB that are activated in response to low pH, low magnesium, high iron and in response to cationic antimicrobial peptides (432). More specifically, PhoP-PhoQ regulates the magnesium regulon, which activates polymyxin resistance under low magnesium conditions. This PhoP-PhoQ system is connected by the small basic protein PmrD. PhoP regulates the activation of PmrD, which can then bind to PmrA and prolong its phosphorylation state, eventually activating the expression of the PmrA-PrmB system to promote lipid A modifications that confer polymyxin resistance. The under-acylation of lipid A increases the polymyxin susceptibility of *K. pneumoniae*, which highlights that the decoration of lipid A with additional fatty acyl chains is important for polymyxin resistance (441, 442).

Gram-negative bacteria naturally shed their OM via OMVs, which are spherical bilayer structures of approximately 20-200 nm in diameter (443). OMVs are believe to serve as delivery vehicles for nefarious bacterial cargo such as virulence factors, antibiotic resistance determinants, toxins and factors that modulate the host immune response to facilitate pathogen evasion (303, 443, 460-462). This underscores the need to understand the compositional differences between OMVs of MDR *K. pneumoniae* clinical isolates and how this relates to their pathogenicity. In the present study, we aimed to perform a comparative analysis of the lipidome of OMVs isolated from of polymyxin-susceptible and -resistant *K. pneumoniae* clinical isolates and to identify key lipid species that are selectively packaged from the OM into the OMV sub-lipidome of the resistant isolates. The obtained data sheds new light on the OMV lipidomes associated with high-level polymyxin resistance in the problematic Gram-negative pathogen *K. pneumoniae*.

4.2 Materials and Methods

4.2.1 Materials

Polymyxin B was supplied by Betapharma (Shanghai, China). All chemicals were purchased from Sigma-Aldrich at the highest research grade; ultrapure water was from Fluka (Castle Hill, NSW, Australia). Stock solutions of polymyxin B (10 mg/L) were freshly prepared in ultrapure water and filtered through 0.22 μm syringe filters (Sartorius, Melbourne, Vic, Australia).

4.2.2 Bacterial isolates and growth conditions

All bacterial strains used in this study are described in **Table 4.1**. Resistance to polymyxin B was defined as MICs of ≥8 mg/L (445). A total of six different *K. pneumoniae* isolates were studied: The clinical isolates *K. pneumoniae* FADDI-KP069 (polymyxin-susceptible strain polymyxin B MIC = 0.5 mg/L; polymyxin-resistant strain polymyxin B MIC >32 mg/L; Both positive for ESBL and KPC carbapenemase) and *K. pneumoniae* BM3 (polymyxin-susceptible strain polymyxin B MIC = 0.5 mg/L; polymyxin-resistant strain polymyxin B MIC ⩾32 mg/L; Both positive for NDM, CTX-M, SHV, TEM, AAC-6'-1B); and a reference strain *K. pneumoniae* ATCC 700721 (polymyxin-susceptible strain polymyxin B MIC = 0.5 mg/L; polymyxin-resistant strain polymyxin B MIC >32 mg/L). The antibiograms of the two clinical isolates are documented in **Table 4.1**. All bacteria were stored at -80°C in tryptone soya broth (TSB, Oxoid, Melbourne, Australia). Prior to experiments, parent strains were sub-cultured onto nutrient agar plates (Medium Preparation Unit, University of Melbourne, Victoria, Australia). Overnight broth cultures were subsequently grown in 5 mL of cation-adjusted Mueller-Hinton broth (CaMHB, Oxoid, West Heidelberg, Victoria, Australia), from which a 1 in 100 dilution was performed in fresh broth to prepare mid-logarithmic cultures according to the optical density at 500 nm (OD_{500nm} = 0.4 to 0.6). All broth cultures were incubated at 37°C in a shaking water bath (180 rpm).

4.2.3 Minimum Inhibitory Concentration (MIC) microbiological assay

MICs were performed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (446). MICs were determined for all isolates in three replicates on separate days using broth microdilution method in cation-adjusted Mueller-Hinton broth (CAMHB) in 96-well polypropylene microtitre plates. Wells were inoculated with 100 µL of bacterial suspension prepared in CaMHB (containing 10^6 colony forming units (cfu) per mL) and 100 µL of CaMHB containing increasing concentrations of polymyxin B (0.25–256 mg/L). The MICs were defined as the lowest concentration at which visible growth was inhibited following 18 h incubation at 37˚C. Cell viability was determined by sampling wells at polymyxin B concentrations greater than the MIC. These samples were diluted in normal saline and spread plated onto nutrient agar. After incubation at 37˚C for 20 h, viable colonies were counted on these plates. The limit of detection was 10 cfu/mL.

Table 4.1 Antibiotic susceptibility and resistance gene profiles for two *K. pneumoniae*

Isolate name		*K. pneumoniae* FADDI-KP069			*K. pneumoniae* BM3
Antibiotic susceptibility		Susceptibility	MIC Value		Susceptibility
	Ampicillin	R	≥32		ND
	Amoxicillin/Clavulanic Acid	R	≥32		ND
	Ticarcillin/Clavulanic Acid	R	≥128		ND
	Piperacillin/Tazobactam	R	≥128		ND
	Cefazolin	R	≥64		ND
	Cefoxitin	R	≥64		ND
	Ceftazidime	R	≥64		ND
	Ceftriaxone	R	≥64		ND
	Cefepime	R	8		ND
	Meropenem	R	≥16		ND
	Amikacin	R	≥64		I
	Gentamicin	R	≥16		R
	Tobramycin	R	≥16		ND
	Ciprofloxacin	R	≥4		R
	Norfloxacin	R	≥16		ND
	Nitrofurantoin	R	256		S
	Trimethoprim	R	≥16		ND
	Trimethoprim/Sulfamethoxazole	R	≥320		ND
	Tetracyclin	ND	ND		R
	Chloramphenicol	ND	ND		S

	Netilmicin	ND	ND		I
	Tigecycline	ND	ND		I
	Fosfomycin	ND	ND		S
	Aztreonam	ND	ND		R
Resistance genes	ESBL	+			ND
	Carbapenemase (metallo or KPC)	+			ND
	NDM-1	ND			+
	CTX-M	ND			+
	CMY-2	ND			-
	SHV	ND			+
	TEM	ND			+
	AAC-6'-1B	+			+

R = Resistant
I = Intermediate
S = Susceptible

4.2.4 Isolation of outer membrane vesicles (OMVs)

Mid-logarithmic cultures (6 L) of each isolate were grown at 37°C with shaking (1800 rpm) and cell free supernatants were collected through centrifugation (15 min at 10,000 × *g*, 4°C). Where indicated, polymyxin B was added to the culture volume at a final concentration of 2 mg/L. The OMV containing supernatants were filtered through 0.22-μm membrane (Sigma-Aldrich) to remove any remaining cell debris., and then concentrated through a tangential filtration concentrator unit (Pall Life Science, Ann Arbor, MI, USA) and collected using 100 kDa Pellicon filtration cassettes (Millipore, Melbourne, Australia). Also, a portion of the supernatant was plated for growth on agar plates overnight at 37°C to make sure that the supernatant is free of bacterial cells. OMVs in the cell-free supernatants were then pelleted down by ultracentrifugation at 150,000 × *g* for 2 h at 4 °C in a Beckman Ultracentrifuge (SW28 rotor). Purified OMVs were concentrated re-suspended in 1 mL sterile PBS and the concentration was determined by Bio-Rad (Gladesville, NSW, Australia) protein assay.

4.2.5 Lipidomics analysis

OMV lipids were extracted with the single-phase Bligh-Dyer method ($CHCl_3$/MeOH/H_2O, 1:3:1, v/v) (463). For further analysis, samples were reconstituted in 100 µL of $CHCl_3$ and 200 µL of MeOH, centrifuged at 14,000 × *g* for 10 min at 4 °C to obtain particle-free supernatants.

LC-MS for lipidomic analysis was conducted on a Dionex U3000 high-performance liquid chromatography system (HPLC) in tandem with a Q-Exactive Orbitrap mass spectrometer (Thermo Fisher, Melbourne, Australia) in both positive and negative mode with a resolution at 35,000. The mass scanning range was from 167 to 2000 *m/z*. The electrospray voltage was set as 3.50 kV and nitrogen was used as collision gas. The Ascentis Express C_8 column (5 cm × 2.1 mm, 2.7 µm, Sigma-Aldrich, 53831-U) was maintained at 40°C, and the samples were controlled at 4°C. The flow rate was 0.2 mL/min at first 24 min, but increased to 0.5 mL/min from 25 min to 30 min. The multi-step gradient started from 100% to 80% mobile phase A over the first 1.5 min, then to 72% mobile phase A at 7 min, over the next 1 min, the gradient changed to 65% mobile phase A, from 8 min to 24 min, the gradient reached a final composition of 35% mobile phase A and 65% mobile phase B. This was followed by a washing step from 65% to 100% mobile phase B over the next 1 min, and maintained for 2 min. A 2-min re-equilibration of the column with 100% A was performed between injections. Untargeted lipidomic analyses were performed through mzMatch (464); and IDEOM (465) (http://mzmatch.sourceforge.net/ideom.php). Raw LC-MS data files were converted to mzXML format through a proteowizard tool, Msconvert. Automated chromatography peaks were picked by XCMS (466), and then converted to peakML files, which were combined and filtered by mzMatch based on the intensity (1000), reproducibility (RSD for all replicates < 0.8), and peak shape (codadw > 0.8). The mzMatch program was used for retrieving intensities for missing peaks and the annotation of related peaks. Unmatched peaks and noises were rejected through IDEOM. The database used in IDEOM included KEGG, MetaCyc and Lipidmaps (467). Univariate statistics analysis was performed using a Welch's T-test (p-value < 0.05), while multivariate analysis was conducted using the metabolomics R package.

4.2.6 Transmission electron microscopy (TEM)

Carbon coated Formvar copper grids were placed on a drop of OMV suspension (1 mg/mL protein) for 5 min then washed three times with PBS and fixed in 1% glutaraldehyde for 4 min.

Grids were then washed three times with PBS, two times with Milli-Q water and stained for 20 s with 4% uranyl acetate. Grids were finally washed with Milli-Q water and incubated on ice for 10 min in methyl-cellulose with 4% uranyl acetate (9:1). Grids were then air dried and viewed with a Tecnai Spirit (T12) transmission electron microscope, and the images were acquired using the TIA software (FEI, Melbourne, Australia).

4.2.7 Dynamic Light Scattering

The particle size of the OMVs was measured by using dynamic light scattering (DLS). OMVs were diluted with PBS to a protein concentration of 0.05 mg/L and the scatter was recorded using a Zetasizer NanoS (Malvern, PA, USA) at 173° with a laser of wavelength 632 nm. Data were analysed with Zetasizer Software (V7.11, Malvern, UK) to obtain the average hydrodynamic radius

4.3 Results and Discussion

4.3.1 Lipidomics analysis of OMVs from polymyxin-susceptible and resistant *K. pneumoniae* isolates

The OMV lipidome from paired polymyxin-susceptible and -resistant strains from two clinical isolates (*K. pneumoniae* BM3 and FADDI-KP069) and a laboratory type strain (*K. pneumoniae* ATCC 700721) were characterised following lipid extraction using LC-MS analysis. Compositional analysis revealed that the OMV lipid composition of all the *K. pneumoniae* strains mostly consisted of glycerophospholipids (~35%), fatty acids (~33%) and sphingolipids (~20%). Similarly, across all three strains the OMV minor lipid components consisted of lipids from the following classes, glyceroipids (~4%), sterol lipids (~3%) and prenol lipids (~4%).

Principle component analysis (PCA) score plots and the heat map revealed significant global lipidomic differences between the OMVs of the polymyxin-susceptible and -resistant *K. pneumoniae* strains (**Figures 4.1 & 4.2**). Notably, following treatment with a clinically relevant

concentration of polymyxin B (2 mg/L) we observed marked global lipidome perturbations in the OMVs of the polymyxin-susceptible *K. pneumoniae* strains; whereas the OMVs of the resistant strains showed moderate global lipidome perturbations in response to polymyxin B treatment of the cells. For univariate analyses, all of the putatively identified lipids were further analysed to reveal those showing at least 2-fold differences ($p < 0.05$, FDR < 0.05, one-way ANOVA test) in relative abundance (**Figure 4.3**). The cluster algorithm and fold-change analysis highlighted that, compared to the untreated controls, polymyxin B treatment (2 mg/L) of the polymyxin-susceptible *K. pneumoniae* ATCC 700721 significantly reduced the phosphatidylcholine, phosphatidylethanolamine and 1-acyl-glycerophosphocholine content of its OMVs. Additionally, the sphingolipids namely, sphingosine, *N*-acyl-sphingosine (ceramide), *N*-acyl-sphinganine(dihydro-ceramide), sphingomyelin, glucosyl-ceramide and lactosyl-ceramide were significantly reduced following polymyxin B treatment (**Figure 4.3A i.**). Moreover, certain saturated fatty acids (e.g. hexadecanoic acid and octadecanoic acid), and polyunsaturated fatty acids (α-linolenic acid and arachidonic acid) were also reduced in the OMVs of the polymyxin B treated susceptible isolate. Polymyxin B treatment of the its paired polymyxin-resistant *K. pneumoniae* ATCC 700721 laboratory isolate significantly increased the content of lysoglycerophosphates, phosphatidylcholines and phosphatidylethanolamines in its OMVs (**Figure 4.3A ii.**). Similarly, to the polymyxin-susceptible ATCC 700721 isolate, most of the glycerophospholipid and fatty acid content of the OMVs isolated from the polymyxin B treated polymyxin-susceptible clinical isolates (*K. pneumoniae* BM3 and FADDI-KP069) were significantly reduced compared to untreated controls (**Figure 4.3B i. and C i.**). In particular, glycerophospholipids (e.g., phosphatidylethanolamines, phosphatidylcholines, lysophosphatidylcholines, and lysoglycerophosphates) were remarkably reduced in response to polymyxin B treatment. In addition, fatty acids (e.g. docosanoic acid, octadecenoic acid and hexadecanoic acid); and sphingolipids (mainly dihydro-ceramides) were also significantly reduced in response to polymyxin B be treatment. In contrast, the majority of glycerophospholipids, fatty acids and sphingolipids content of OMVs isolated from their paired

polymyxin-resistant *K. pneumoniae* BM3 and FADDI-KP069 isolates were significantly increased in response to polymyxin B treatment (**Figure 4.3B ii. and C ii.**). Notably, all of the polymyxin B-resistant strains secreted OMVs significantly are richer in glycerophospholipids, fatty acids, lysoglycerophosphates and sphingolipids compared to polymyxin B-susceptible isolates even when grown in the absence of polymyxin B (**Figure 4.4**). Glycerophospholipids, fatty acids, glycerolipids and sphingolipids play a crucial role in maintain OM integrity, bacterial survival and pathogenesis (468). Phospholipids (including glycerophospholipids) are essential components of bacterial membranes and they are responsible for maintaining membrane integrity and the selective permeability of the OM (469); they contribute to cationic peptide resistance, protect bacteria from osmotic stress and regulate flagellum-mediated motility (470). In addition, sphingolipids are essentially involve in maintaining normal bacterial growth and membrane integrity; and trigger bacterial pathogenesis via induction of the host immune system (471).

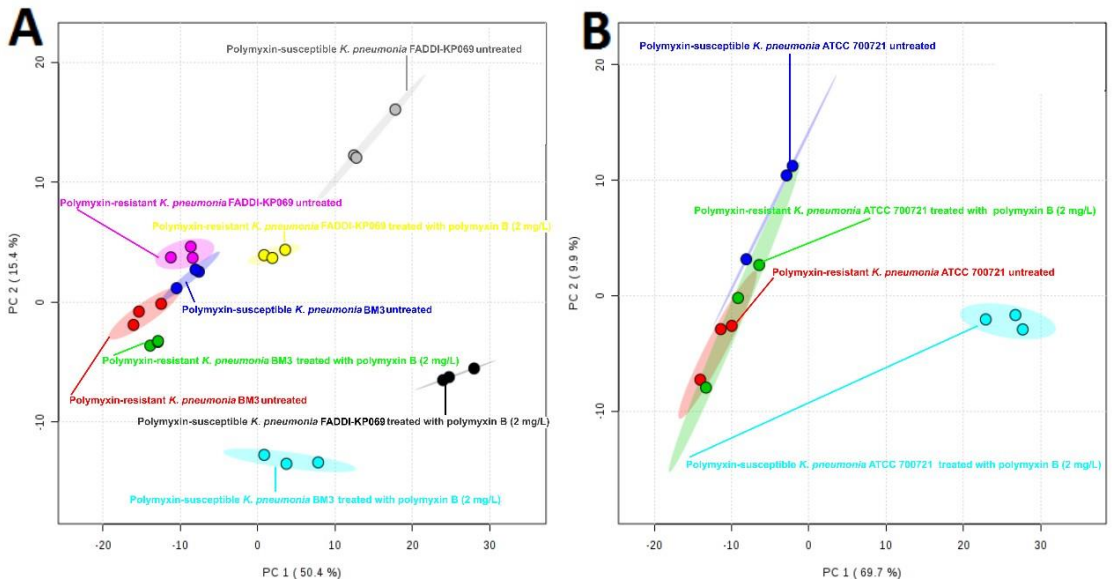

Figure 4.1 Principal component analysis (PCA) score plot for OMVs isolated from polymyxin-susceptible and -resistant *K. pneumoniae* isolates. (**A**) PCA score plot for the two clinical isolates. Polymyxin-resistant *K. pneumoniae* BM3 untreated (red); polymyxin-resistant *K. pneumoniae* BM3 treated with polymyxin B (2 mg/L) (green); polymyxin-susceptible *K. pneumoniae* BM3 untreated (blue); polymyxin-susceptible *K. pneumoniae* BM3 treated with polymyxin B (2 mg/L) (cyan); polymyxin-resistant *K. pneumoniae* FADDI-KP069 untreated (purple); polymyxin-resistant *K. pneumoniae* FADDI-KP069 treated with polymyxin B (2 mg/L) (yellow); polymyxin-susceptible *K. pneumoniae* FADDI-KP069 untreated (grey); polymyxin-susceptible *K. pneumoniae* FADDI-KP069 treated with polymyxin B (2 mg/L) (black). (**B**)

PCA score plot for the paired *K. pneumoniae* ATCC 700721 laboratory type isolates. Polymyxin-resistant *K. pneumoniae* ATCC 700721 untreated (red); polymyxin-resistant *K. pneumoniae* ATCC 700721 treated with polymyxin B (2 mg/L) (green); polymyxin-susceptible *K. pneumoniae* ATCC 700721 untreated (blue); polymyxin-susceptible *K. pneumoniae* ATCC 700721 treated with polymyxin B (2 mg/L) (cyan). Each data point represents three biological replicates.

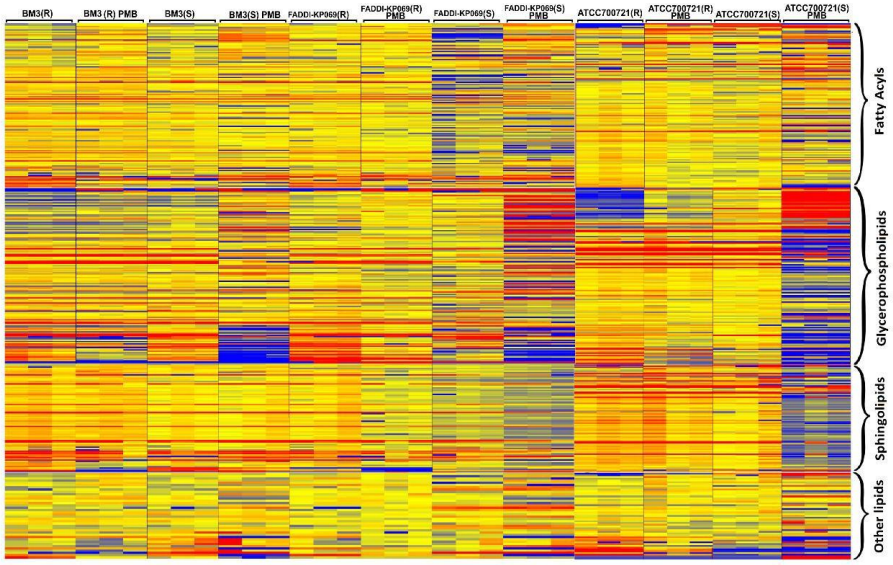

Figure 4.2 The heat map illustrates the relative peak intensity of lipids within each class in the OMVs of the paired polymyxin-susceptible and -resistant *K. pneumoniae* isolates. (R) = polymyxin-resistant; (S) = Polymyxin-susceptible. Colours indicate relative abundance of lipidomes based on the relative peak intensity (red = high, yellow = no change, blue = undetectable).

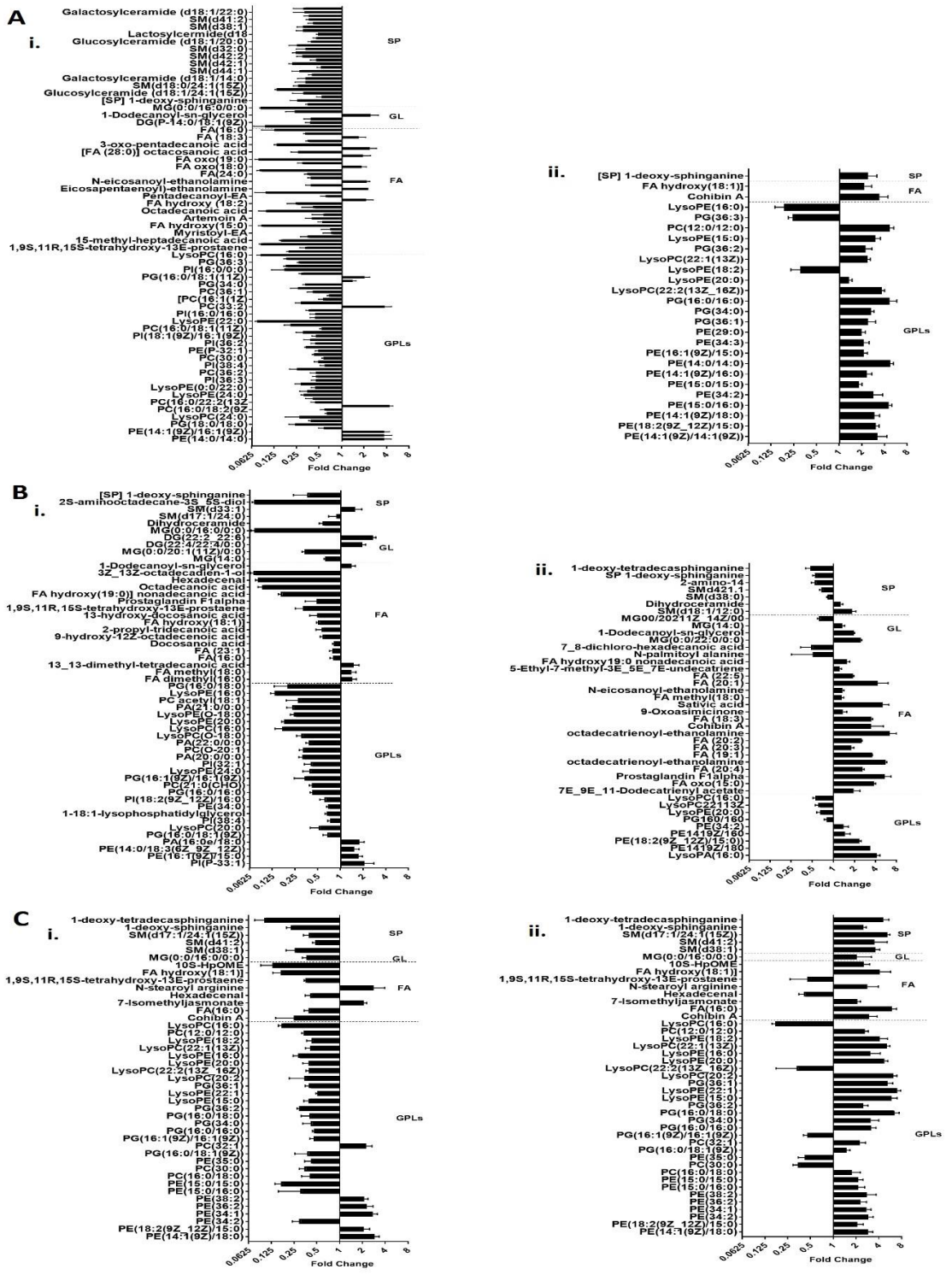

Figure 4.3 Lipidomic perturbations of OMVs isolated from polymyxin-susceptible and -resistant *K. pneumoniae* isolates. Fold-change of lipids relative to the untreated control cells, in OMVs of the polymyxin-susceptible (**i**) and -resistant (**ii**) strains of paired *K. pneumoniae* isolates in response to polymyxin B treatment (2 mg/L). (**A**) *K. pneumoniae* ATCC 700721. (**B**) *K. pneumoniae* BM3 and (**C**) *K. pneumoniae* FADDI-KP069. GPLs = glycerophospholipids; FA = fatty acids; GL = glycerolipids; SP = sphingolipids.

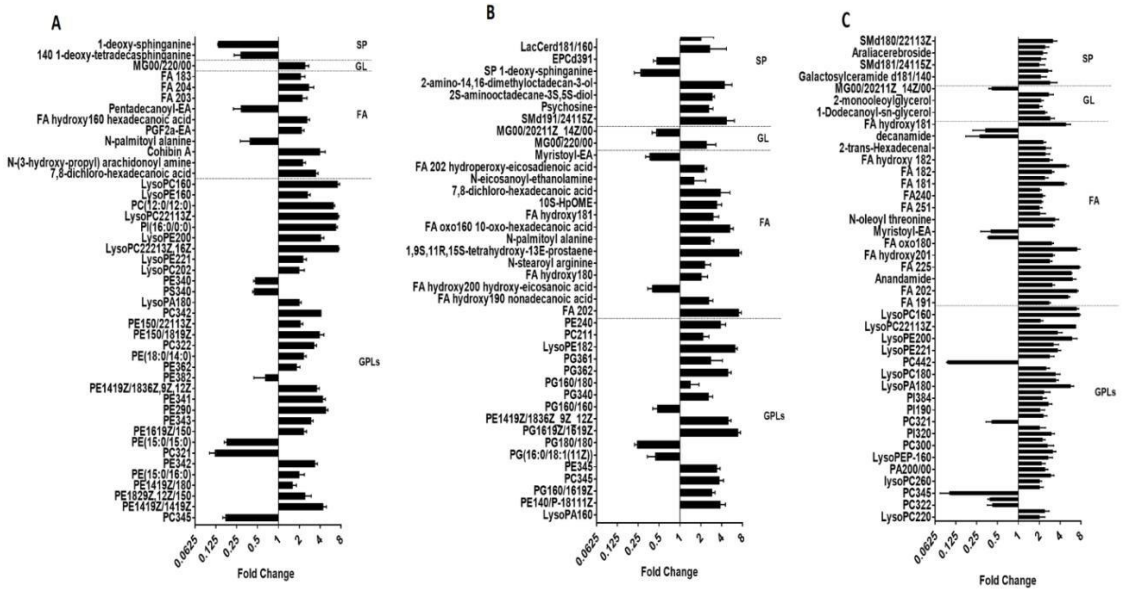

Figure 4.4 Major differences in the lipid abundance between the OMVs of paired polymyxin-susceptible and -resistant *K. pneumoniae* isolates. The differences are expressed as the fold-change in the OMV lipids of the paired susceptible vs. resistant *K. pneumoniae* isolates. All cultures were grown in the absence of polymyxins. (**A**) *K. pneumoniae* ATCC 700721. (**B**) *K. pneumoniae* BM3 and (**C**) *K. pneumoniae* FADDI-KP069. GPLs = glycerophospholipids; FA = fatty acids; GL = glycerolipids; SP = sphingolipids.

4.3.2 Transmission electron microscopy imaging and dynamic light scattering size estimation of *K. pneumoniae* OMVs

Dynamic light-scattering (DLS) analysis revealed that the average hydrodynamic radius of the OMVs derived from the *K. pneumoniae* ATCC 700721 polymyxin-susceptible isolate is ~90.6 nm; the profile was symmetrical and the OMV scatter ranged from ~30–500 nm **(Figure 4.5A)**. The average hydrodynamic radius of the OMVs isolated from the paired *K. pneumoniae* ATCC 700721 polymyxin-resistant isolate was ~141 nm and the OMV scatter ranged from ~30 to 1000 nm **(Figure 4.5C)**, which indicates that the resistant isolae sheds larger OMVs than the susceptible one. Polymyxin B treatment (2 mg/L) of the *K. pneumoniae* cells resulted in the production of OMVs with slightly larger average particle size in both the susceptible (average diameter ~124 nm, OMV scatter ~30–900 nm; **Figure 4.5B**) and resistant (average hydrodynamic radius ~154 nm, OMV scatter ~30–1500 nm; **Figure 4.5D**) strains. Notably, the

OMV scatter profile in the resistant strain is asymmetrical, with and without polymyxin B treatment. In line with the DLS data (472, 473), TEM imaging of *K. pneumoniae* OMVs revealed a similar size distribution wherein the polymyxin-resistant *K. pneumoniae* ATCC 700721 strain produced larger OMVs than the susceptible strain **(Figure 4.6)**. Moreover, the OMVs isolated from the polymyxin-resistant isolate stained darker with the TEM contrast reagent uranyl acetate, which enhances the contrast by interaction with lipids; in line with the lipidomics findings, this would suggest that the OMVs of the resistant strains contain more lipids. Similarly, in Salmonella enterica, LPS remodelling in the OM in response to polymyxins or other environmental PhoP/Q–PmrA/B activating conditions, has been shown to stimulate the biogenesis of larger-diameter OMVs (303, 460-462).

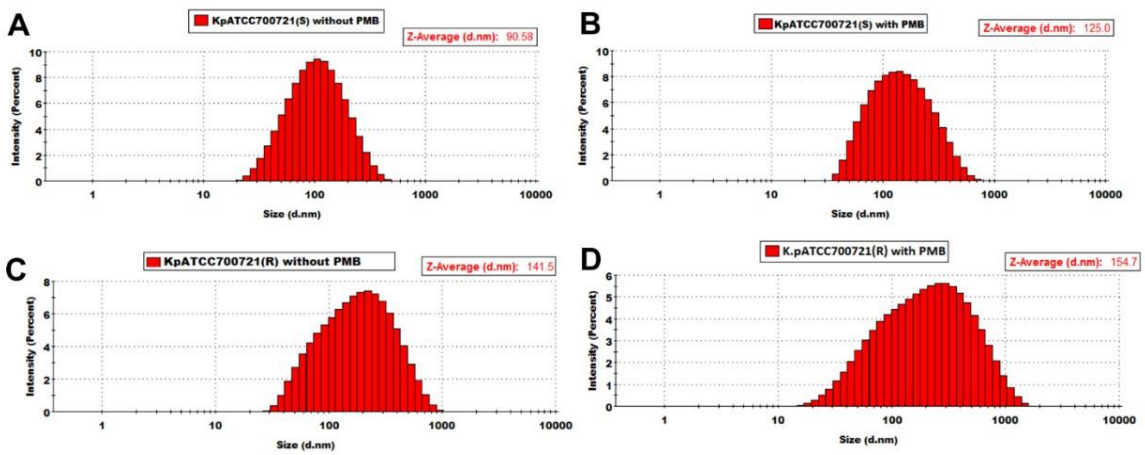

Figure 4.5 Size distribution measured by dynamic light scattering of OMVs isolated from paired polymyxin-susceptible and -resistant strains of *K. pneumoniae* ATCC 700721. OMVs isolated from the polymyxin-susceptible *K. pneumoniae* ATCC 700721 (**A**) without polymyxin B treatment and (**B**) with polymyxin B (2 mg/L) treatment. OMVs isolated from the polymyxin-resistant *K. pneumoniae* ATCC 700721 (**C**) without polymyxin B treatment and (**D**) with polymyxin B (2 mg/L) treatment. The data are is presented as the mean SUV size±SD of four independent measurements. Polymyxin-resistant strain released larger OMVs than susceptiple strain. Moreover, both susceptible and resistatnt strains produced

OMVs larger in particle size compared to OMVs derived from untreated ones.

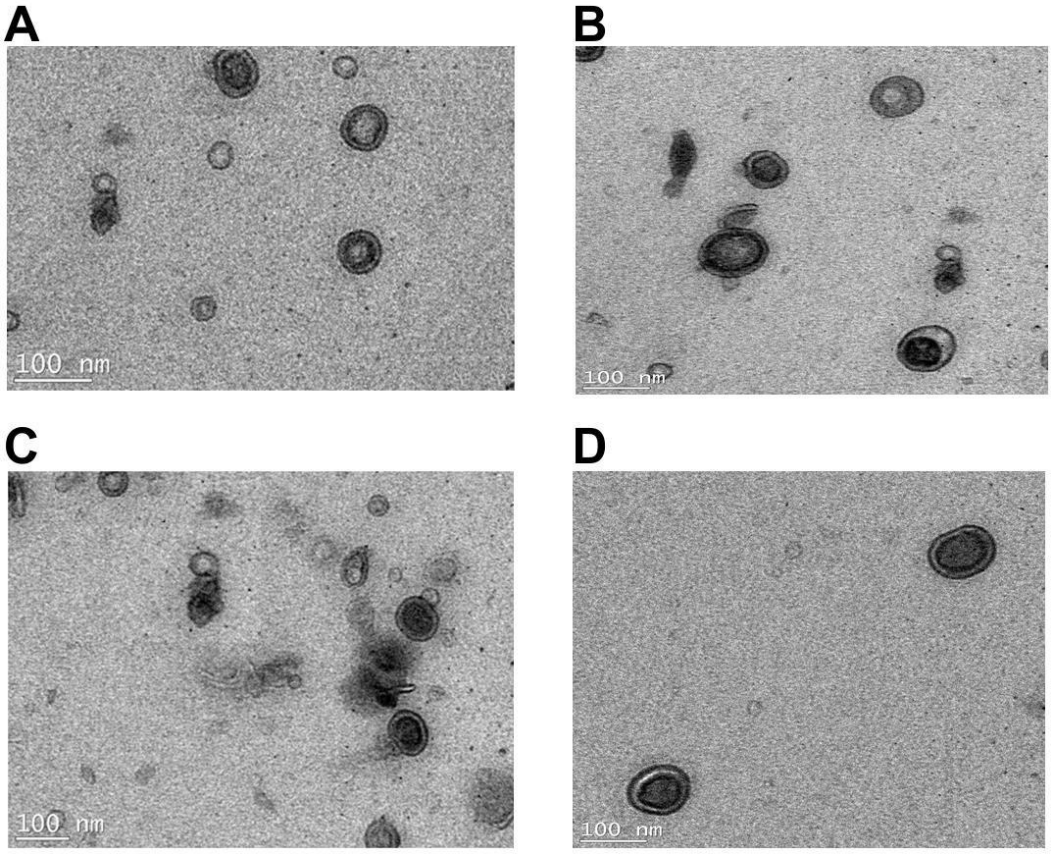

Figure 4.6 Transmission electron microscopy images of OMVs isolated from paired polymyxin-susceptible and -resistant strains of *K. pneumoniae* ATCC 700721. (**A**) OMVs from untreated *K. pneumoniae* ATCC 700721 (susceptible). (**B**) OMVs from polymyxin B (2 mg/L) treated *K. pneumoniae* ATCC 700721 (susceptible). (**C**) OMVs from untreated *K. pneumoniae* ATCC 700721 (resistant). (**D**) OMVs from polymyxin B (2 mg/L) treated *K. pneumoniae* ATCC 700721 (resistant).

4.4 Conclusions

In this study, we show that polymyxin B treatment of the susceptible *K. pneumoniae* strains significantly reduced the glycerophospholipid, fatty acid, lysoglycerophosphate and sphingolipid content of their OMVs, compare to the untreated control. On the other hand, in the OMVs of their paired polymyxin-resistant strains these lipids were increased both intrinsically and in response to polymyxin B treatment. In view of these findings, it is reasonable to hypothesize that the OM remodelling associated with polymyxin- resistance in *K. pneumoniae* entails fortifying the membrane with increased glycerophospholipids, fatty

acids, lysoglycerophosphates and sphingolipids, which are lipids to which polymyxins cannot avidly bind. It is important to mention that polymyxins primarily target the lipid A in the Gram-negative OM—hence their narrow spectrum of activity against Gram-negative bacteria that do not express LPS. These OM changes may be accompanied by the modification of the lipid A with cationic moieties and/or a reduction in the lipid A content, which, together with the increased content of the aforementioned lipids, serve to make the *K. pneumoniae* OM and OMVs more impervious to polymyxin attack.

Chapter 5: A comparative study of outer membrane proteome between a paired colistin-susceptible and extremely colistin-resistant *Klebsiella pneumoniae* strains

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A Comparative Study of Outer Membrane Proteome between Paired Colistin-Susceptible and Extremely Colistin-Resistant *Klebsiella pneumoniae* Strains

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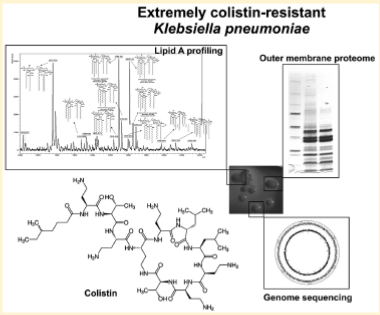
Supporting Information

ABSTRACT: In the present report we characterized the outer membrane proteome, genomic, and lipid A remodelling changes following the evolution of a colistin-susceptible *K. pneumoniae* ATCC 13883 strain into an extremely colistin-resistant strain. Lipid A profiling revealed the outer membrane of the colistin-susceptible strain is decorated primarily by *hexa*- and *hepta*-acylated lipid A species and a minor *tetra*-acylated species. In the lipid A profile of the extremely colistin-resistant strain, in addition to the aforementioned lipid A species, the obligatory 4-amino-4-deoxy-L-arabinose modification of the *hexa*-acylated lipid A was detected. Comparative genomic analysis revealed that the *mgrB* gene of the colistin-resistant strain is inactivated by a single nucleotide insertion which produces a frame-shift, resulting in premature termination. We also detected two synonymous mutations in the two-component system genes *phoP* and *phoQ*. Comparative profiling of the outer membrane proteome of each strain revealed that outer membrane proteins from bacterial stress response, glutamine degradation, pyruvate, aspartate, and asparagine metabolic pathways were over-represented in the extremely colistin-resistant *K. pneumoniae* ATCC 13883 strain. In comparison, in the sensitive strain, outer membrane proteins from carbohydrate metabolism, H^+-ATPase, cell division, and peptidoglycan biosynthesis were over-represented. Notably, there were no discernible differences between the OmpK35 and OmpK36 major outer membrane porins between the polymyxin-susceptible and -resistant strains suggesting porin deficiency is not involved in the colistin resistance in the ATCC 13883 strain. These findings shed new light on the outer membrane remodelling events accompanying the development of extremely high levels of colistin resistance in *K. pneumoniae*.



KEYWORDS: *colistin, Klebsiella pneumoniae, outer membrane proteome*

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5.1 Introduction

Over the last decade, extremely-drug resistant (XDR) *K. pneumoniae* has emerged as one of the deadliest Gram-negative 'superbugs' (413-415). *K. pneumoniae* is responsible for numerous lethal nosocomial outbreaks (e.g. pneumonia and bloodstream infections), particularly in immune-compromised patients (416). Carbapenem resistance in *K. pneumoniae* mediated by carbapenemase was initially reported in 1996 in the New York City and has spread to most global centres (417, 418). In their 2013 Threat Report on Antimicrobial Resistance, the US Centres for Disease Control and Prevention prioritized Carbapenem-Resistant *Enterbacteriaceae* as an urgent threat (i.e. the highest level) (474). Polymyxins (i.e. colistin and polymyxin B, **Figure 5.1A**) are increasingly used as the last-line therapy against XDR *K. pneumoniae* (15). Indeed, considerable *in vitro* activity against *K. pneumoniae* strains has been demonstrated (136); 98.2 % of general clinical strains of *K. pneumoniae* are susceptible to polymyxin B and colistin (420-425). Worryingly, XDR strains which are resistant to several antibiotics including the polymyxins have emerged (426, 427). The emergence of these XDR strains demands a greater appreciation of the mechanism(s) of polymyxin resistance employed by *K. pneumoniae* to assist targeted drug discovery strategies against these problematic XDR Gram-negative pathogens.

The Gram-negative OM constitutes a permeability barrier to various noxious substances for the bacterial cell, such as antimicrobials (156, 428). This complex asymmetrical structure comprises an inner phospholipid leaflet, as well as an outer leaflet that predominantly contains proteins, phospholipids and LPS, (**Figure 5.1B**). Structurally, LPS is composed of three domains; (i) the variable *O*-antigen chain (encompassing repeated saccharide units); (ii) a core-oligosaccharide region and (iii) the conserved lipid A moiety. Lipid A is embedded within the OM leaflet, functioning as a hydrophobic anchor for the LPS molecule (475). Additionally, *K.*

pneumoniae commonly expresses a capsular polysaccharide that coats the OM, the expression levels of which have been related to polymyxin susceptibility (429-432).

The precise mechanism of action of polymyxins remains contentious, and based on biophysical studies a number of models have been put forward (476). The consensus view is that polymyxins are OM active antibiotics and that lipid A is the primary polymyxin target (476). A well accepted model termed the 'self-promoted uptake' pathway, purports that the amphipathic nature of polymyxins is crucial to enable uptake of the polymyxin molecule across the OM barrier (477-479). In the self-promoted uptake model, protonation of free γ-amines on the Dab residues of polymyxins at physiological pH, provides a means of electrostatic attraction to anionic 1′- and 4′-phosphates of lipid A (476). The resultant displacement of divalent cations that stabilize the LPS leaflet, allows the hydrophobic *N*-terminal fatty-acyl tail, and D-Phe-L-Leu or D-Leu-L-Leu motifs (of polymyxin B and colistin, respectively) to be inserted into the OM fatty acyl layer. Notably, polymyxin nonapeptide which has the *N*-terminal fatty acyl-Dab^{1} removed is inactive, which highlights these hydrophobic interactions with lipid A acyl chains play an important role in the disruption of the OM (476, 477). Subsequent events are not completely understood, however, polymyxin-mediated fusion of the inner leaflets of the outer- and cytoplasmic-membranes surrounding the periplasmic space, is believed to induce phospholipid exchange, resulting in cell wall disruption which culminates in cell death (480-482). Albeit, the relationship between bacterial killing activity, polymyxin resistance and OM remodelling events that perturb the ability of polymyxins to interact with the OM components, remains poorly characterized in *K. pneumoniae,* and therefore warrants further investigation. Particularly, a proteomic approach has yet to be applied to the OM of polymyxin-resistant *K. pneumoniae,* where most studies have focused on the roles of LPS and the capsule (429, 431, 441). OMPs play a vital role in bacterial pathogenesis and are involved

in adhesion, penetration, complement activation, virulence proliferation and antibiotic resistance (428, 430, 483, 484). Compared to *Escherichia coli*, very little is known regarding the OM proteome of *K. pneumoniae*, let alone its relation to antibiotic resistance.

In the present study, we performed a comparative bioinformatic and proteomic analysis for the OM proteome and lipid A profiles of a paired colistin-susceptible and extremely colistin-resistant *K. pneumoniae* ATCC 13883 strains. The genome of both strains was sequenced, assembled and scanned to generate a comprehensive inventory of polymyxin resistance determinants in each strain. The obtained data sheds new light on the OM remodelling associated with extremely high levels of colistin resistance in *K. pneumoniae*.

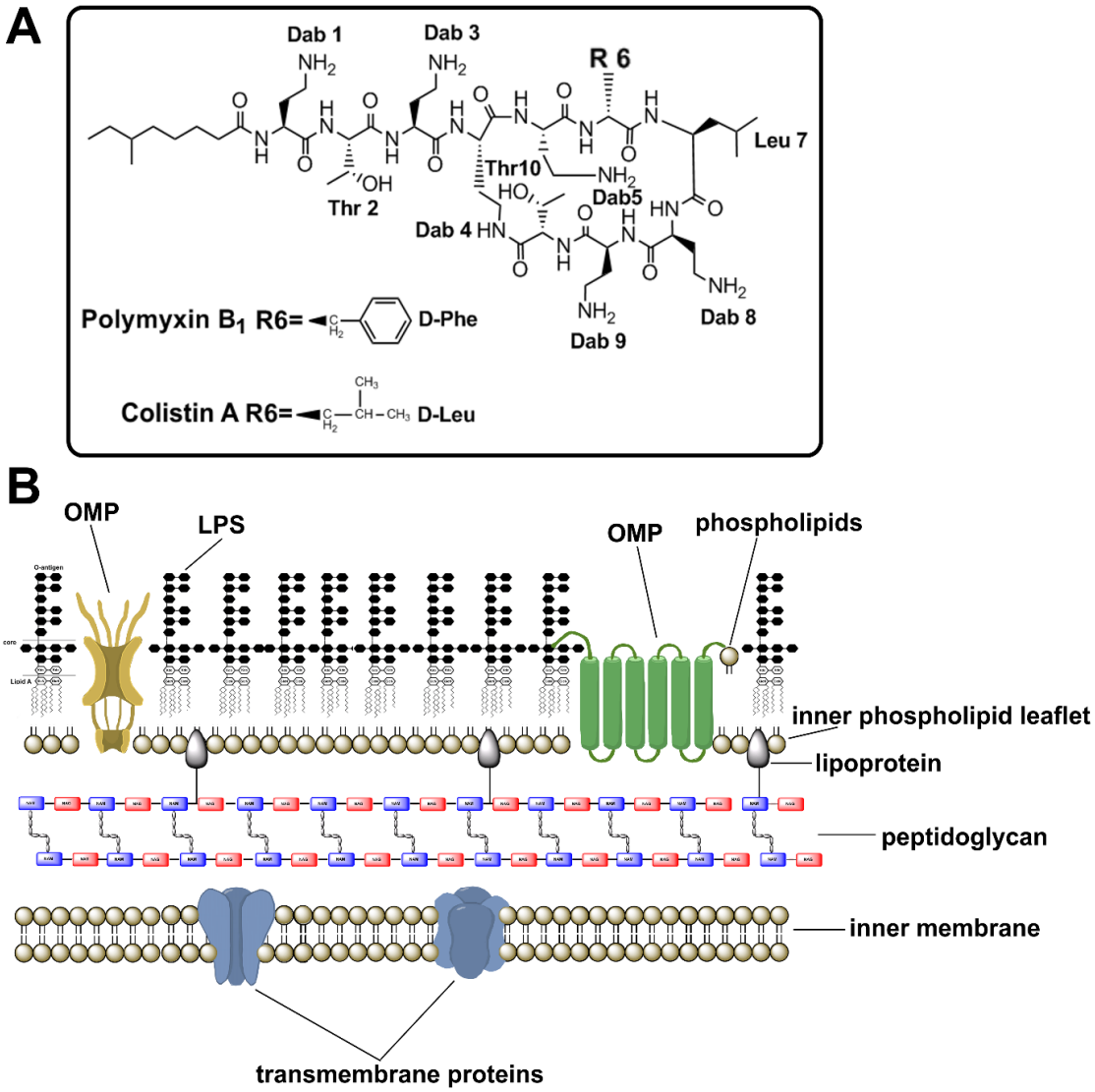

Figure 5.1 (**A**) Chemical structure of colistin and polymyxin B. Leu: leucine; Phe: phenylalanine; Dab: α,γ-diaminobutyric acid. (**B**) Architecture of the Gram-negative cell envelope.

5.2 Materials and Methods

5.2.1 Materials

Colistin sulphate (Batch 20120719) was supplied by Betapharma (Shanghai, China). All chemicals were purchased from Sigma-Aldrich at the highest research grade, ultrapure water was from Fluka (Castle Hill, NSW Australia), Tris from ICN biochemicals (Castle Hill, NSW,

Australia) and the precast-SDS gels were from (NuSep Ltd, Lane Cove, NSW, Australia). Stock solutions of colistin (10 mg/L) were prepared in Milli-Q™ water (Millipore, North Ryde, NSW, Australia) and filtered through 0.22 µm syringe filters (Sartorius, Melbourne, Vic, Australia).

5.2.2 Bacterial strains and growth conditions

K. pneumoniae ATCC 13883 was obtained from the American Type Culture Collection (Manassas, VA, USA) and used as a reference strain (colistin minimum inhibitory concentration (MIC) = 0.5 µg/mL). A paired colistin-resistant strain (colistin MIC > 256 µg/mL) was selected from this reference strain in the presence of 100 µg/mL colistin. All bacteria were stored at -80ºC in tryptone soya broth (TSB, Oxoid Australia). Prior to experiments, parent strains were sub-cultured onto nutrient agar plates (Medium Preparation Unit, University of Melbourne, VIC, Australia). Overnight broth cultures were subsequently grown in 5 mL of cation-adjusted Mueller-Hinton broth (CaMHB, Oxoid, West Heidelberg, VIC, Australia), from which a 1 in 100 dilution was performed in fresh broth to prepare mid-logarithmic cultures according to the optical density at 500 nm (OD_{500nm} = 0.4 to 0.6). All broth cultures were incubated at 37°C in a shaking water bath (180 rpm).

5.2.3 Minimum Inhibitory Concentration (MIC) microbiological assay

MICs for each strain were determined by the broth microdilution method (485). Experiments were performed with CaMHB in 96-well polypropylene microtitre plates. Wells were inoculated with 100 µL of bacterial suspension prepared in CaMHB (containing 10^6 colony forming units (cfu) per mL) and 100 µL of CaMHB containing increasing concentrations of polymyxins. The MICs were defined as the lowest concentration at which visible growth was

inhibited following 18 h incubation at 37°C. Cell viability was determined by sampling wells at polymyxin concentrations greater than the MIC. These samples were diluted in normal saline and spread plated onto nutrient agar. After incubation at 37°C for 20 h, viable colonies were counted on these plates. The limit of detection was 10 cfu/mL.

5.2.4 Isolation of outer membrane proteins (OMPs)

OMPs were extracted as described previously with minor modifications to the original protocol (486). Briefly, cells from a 600 mL culture of *K. pneumoniae* ATCC 13883 were pelleted by centrifugation (3200 × *g* for 10 min at 4°C) and resuspended in 30 mL 10 mM *N*-2 hydroxy ethyl piperazine-*N*'-2-ethane sulfonic acid (HEPES), pH 7.4. The cells were lysed by sonication for 10 minutes (pulse at 5 sec/5 s on ice) and then centrifuged at 3200 × *g* for 10 min at 4°C to remove the cell debris. The supernatant was combined with 7.5 mL of 2% (w/v) *N*-lauroylsarcosine and incubated for 20 min at 37°C for 30 min with shaking. The detergent solubilized OMPs were recovered by high speed centrifugation at 75 000 x *g* for 1 h at 4°C (Beckman, RA 25.50 rotor) and the pellet was resuspended in 30 mL 10 mM HEPES, pH 7.4 and combined with 7.5 mL of 2% (w/v) *N*-lauroylsarcosine and incubated for 20 min at 37°C for 30 min with shaking. The OMPs were recovered by high speed centrifugation at 75 000 x *g* for 1 h at 4°C. Following this final ultracentrifugation, the pellet was resuspended in 1500 µL of 10 mM HEPES, pH 7.4 and stored at -20°C.

5.2.5 SDS-PAGE

Protein concentration was measured by the Bradford assay and equalized for gel loading. SDS-PAGE was carried out on 4-20% polyacrylamide Tris-Glycine gels and OMPs were detected by Coomassie Blue staining. Fractionation of the OMPs was achieved by dissecting uniform

pieces of the gel (1 mm wide bands). Individual polyacrylamide gel plugs were washed 5 × with 25 mM ammonium bicarbonate in 50% methanol. The gel slices were shrunk by leaving them overnight in pure acetonitrile (ACN). ACN was then removed and the gel plug allowed to air dry at room temperature. The plugs were reconstituted with 50 mM ammonium bicarbonate containing 800 ng of trypsin (Promega, Annandale, NSW Australia), and left at 37^{o}C overnight. The resulting peptides were extracted by double application of 20 µL of 50% ACN /0.1 % trifluoracetic acid and the extracts combined.

5.2.6 Ultra High Pressure Liquid Chromatography coupled with Mass Spectrometry

For all experiments, an Ultimate3000 ultra high-pressure liquid chromatography system (Dionex, Castle Hill, Sydney) was used equipped with a ternary low pressure mixing gradient pump (LPG-3600), a membrane degasser unit (SRD-3600), a temperature controlled pulled-loop autosampler (WPS-3000T) and a temperature controlled column oven with flow manager (FLM-3100). The LC experiments were performed using the "pre-concentration" set-up under the following conditions: Nano-column C_{18} PepMap100, 75 µm ID × 150 mm, 3 µm, 100 Å; mobile phase A: 99.9% water + 0.1% FA (v/v, formic acid); mobile phase B: 20/80 water/ACN (v/v) + 0.08 % FA; flow rate nano-column, 400 nL/min; gradient, 2-40% B over 45 min, 90% B for 5 min, 4% B for 30 min; loop size, 5 µL; injection volume, 4 µL (FullLoop) by User Defined Program. The oven was set to 35°C. For the identification of peptides, CID experiments were performed using an AmaZon ETD Ion Trap (Bruker Daltonik GmbH, Australia) equipped with an online-nano-sprayer spraying from a 0.090 mm i.d. and 0.02 mm i.d. fused silica capillary. Fine tuning was achieved using the smart parameter setting option (SPS) for 900 *m/z*, compound stability 60%, and trap drive level at 100% in normal mode resulted in the following mass spectrometric parameters: dry gas temperature, 180°C; dry gas,

4.0 L min^{-1}; nebulizer gas, 0.4 bar; electrospray voltage, 4500 V; high-voltage end-plate offset, -200 V; capillary exit, 140 V; trap drive, 57.4; funnel 1 in 100 V, out 35 V and funnel 2 in 12 V, out 3.3 V; ICC target, 500000; maximum accumulation time, 50 ms. The sample was measured with the Enhanced Scan Mode at 8100 *m/z* per second (which allows monoisotopic resolution up to four charge stages), polarity positive, scan range from *m/z* 100-3000, 5 spectra averaged and rolling average of 2. Acquired tandom mass spectra were processed in Data Analysis 4.0; deconvoluted spectra were further analyzed with BioTools 3.2 software and submitted to Mascot database search (Mascot 2.2.04, Swissprot database (546,439 sequences; 194,445,396 residues, release date 23 Mar 2015). The species subset was set at *K. pneumoniae* ATCC 13883, parent peptide mass tolerance +/- 0.4 Da, fragment mass tolerance +/- 0.4 Da; enzyme specificity trypsin with 2 missed cleavages considered. The following variable modifications have been used: Deamidation (NQ) and Oxidation (M) and carrbomidiomethyaltion (C).

5.2.7 Proteomics analysis

The derived MS datasets on the 3D-trap system were combined into protein compilations using the Protein Extractor functionality of Proteinscape 2.1.0 573 (Bruker Daltonics, Bremen, Germany), which conserved the individual peptides and their scores, while combining them to identify proteins with much higher significance than achievable using individual searches. To exclude false positive identifications, peptides with Mascot scores below 40 (chosen on the basis of manual evaluation of the MS/MS data of peptides with scores below this number) were rejected. The identified protein sequences were manually validated in BioTools (Bruker Daltonics, Bremen, Germany) on a residue-by residue basis using the raw data to ensure accuracy. In brief, the ion series were inspected to ensure: the peaks being selected were not

simply base-line, the accuracy between the residues was less than 0.15 Da and that, preferentially, an overlapping ion series was found, as explained in greater detail in the Results section. When run against a reversed database, and the spectra manually interpreted in a similar fashion, we found a false discovery rate of less than 0.1%.

5.2.8 GO term enrichment

Since each protein was annotated using the genome of type strain MGH 78578 as reference, the gene ontology (GO) terms were crossreferred using MGH 78578 PATRIC, (487) identifiers and the GO annotations provided by UniProt (447). The subcellular localisation of each protein was predicted using PSORTb 3.0 (488). GO term enrichment using Fisher's exact test was implemented in R with FDR<0.05.

5.2.9 Metabolic pathway analysis

The corresponding BioCyc,(489) identifier of each protein was crossreferred using PATRIC,(487) identifiers and the annotation from UniProt (447). The protein associated metabolic reactions were highlighted in BioCyc Cellular Overview Diagram (490). Enrichment analysis of the metabolic pathway classifications was performed using BioCyc embedded functions with FDR<0.1.

5.2.10 Lipid A isolation and structural elucidation

The *K. pneumoniae* ATCC 13883 strains were subcultured onto nutrient agar plates. The colistin resistant strain was maintained on agar plates containing colistin at 50 µg/mL. Overnight (20 h) cultures were subsequently grown in 5 mL of CaMHB at 37°C, from which

a 1:100 dilution was performed into fresh media to prepare a 100 mL culture which was then grown to an OD_{600nm} = 0.8. Lipid A was isolated by mild acid hydrolysis (491). In brief, the bacterial cells (OD_{600nm}= 0.8) from 100 mL of liquid culture were harvested via centrifugation at 3,220 ×*g* for 20 min, and then washed twice with 5 mL of PBS. The cells were re-suspended in 4 mL of PBS, then chloroform (5 mL) and methanol (10 mL) were then added to the suspension, producing a single-phase Bligh-Dyer mixture (chloroform/methanol/water, 1:2:0.8, v/v) (492). The mixture was centrifuged at 3,220 × *g* for 15 min to remove the supernatant. The LPS pellet was washed once with chloroform/methanol/water (1:2:0.8, v/v) and resuspended in 5.4 mL of hydrolysis buffer (50 mM sodium acetate pH 4.5, 1% SDS), and incubated in a boiling water bath for 45 min. To extract the lipids after hydrolysis, the SDS solution was converted into a double-phase Bligh-Dyer mixture by adding 6 mL of chloroform and 6 mL of methanol, forming a chloroform/methanol/water (1:1:0.9, v/v) mixture (492). The lower phase containing lipid A was finally extracted. Samples were dried and stored at -20°C for further analysis. Structural analysis of lipid A was performed by electrospray ionization (ESI) tandem mass spectrometry in negative mode performed on a Q-Exactive Hybrid Quandrupole-Orbitrap Mass Spectrometer (Thermo Fisher).

5.2.11 Genome sequencing

Genomic DNA was purified using a Qiagen genomic DNA extraction kit. Deploying a whole genome shotgun strategy, the genomic DNA was sheared to fragments of approximately 500 base pairs (bp); sequencing libraries were then prepared from the sheared DNA according to Illumina protocols for paired-end sequencing and run on an Illumina GAIIx. The 150 bp, paired-end read set was *de novo* assembled using Velvet (493).

5.3 Results and Discussion

5.3.1 OMP proteome differences between paired colistin-susceptible and extremely colistin-resistant *K. pneumoniae* ATCC 13883 strains

There exists a dearth of knowledge regarding the OMP of polymyxin-resistant *K. pneumoniae* strains. Campos *et. al.,* (431) showed that resistance to cationic antimicrobial peptides in *K. pneumoniae* was due to, in part by the thickness of capsular polysaccharide material coating the surface, albeit recent studies have also shown that polymyxin susceptibility was not altered in a non-encapsulated *K. pneumoniae* mutants (432, 441). This would suggest that besides the capsule, additional components that constitute the OM serve an important barrier function against cationic antibiotics such as the polymyxins (156, 428). The OMPs of antibiotic resistant (494) *K. pneumoniae* have not been comprehensively studied and there is no established nomenclature (495-497). However, it is known that resistance to polymyxin B and β-lactam antibiotics is associated with the loss of either of the 35 and 36 kDa doublet, respectively termed OmpK35 and OmpK36.(430, 483, 484, 498-500). The OMP profile of each strain was initially resolved 1D by SDS-PAGE, which revealed five prominent bands with molecular masses of approximately 46, 36 (OmpK36), 35 (OmpK35), 20 and 12 kDa (**Figure 5.2**). There were no discernible differences between the OmpK35 and OmpK36 of the polymyxin-susceptible and resistant strain of *K. pneumoniae* ATCC 13883. This suggests OmpK35 and OmpK36 porin deficiency is not involved in the polymyxin resistance mechanism(s) employed by this strain.

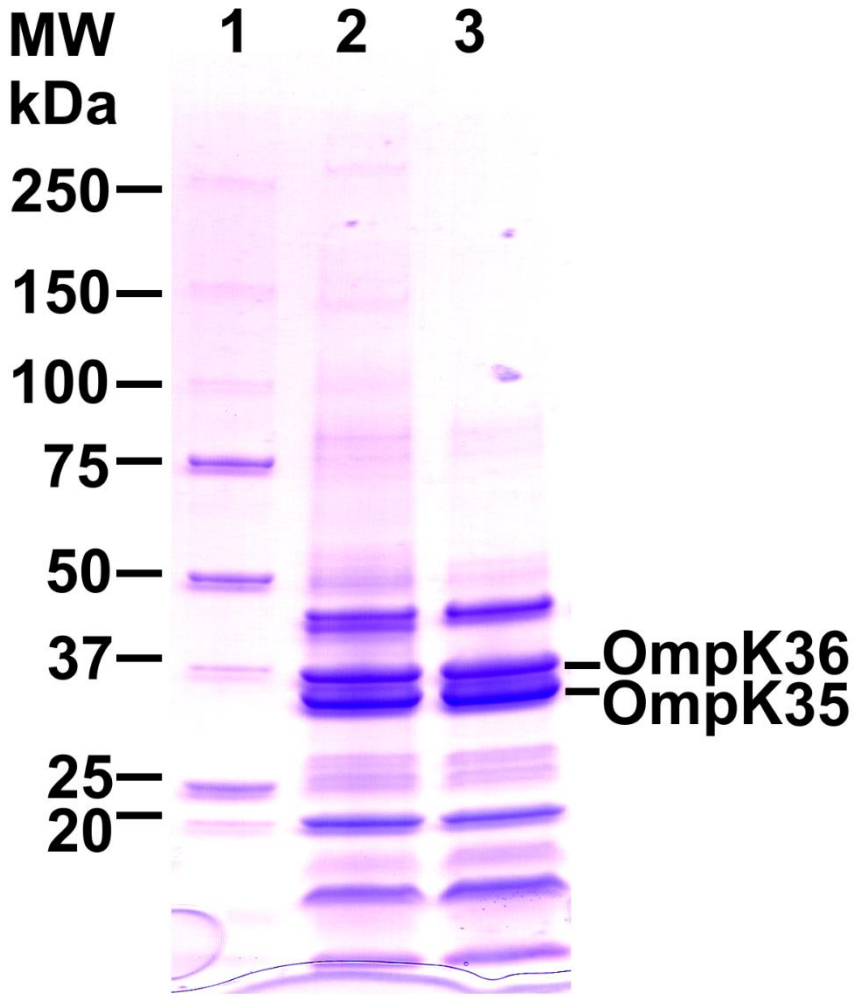

Figure 5.2 OMP profile of the paired polymyxin-susceptible and extremely polymyxin-resistant *K. pneumoniae* ATCC 13883 strains resolved on 4-15% SDS-PAGE gels and visualized by Coomassie Blue staining. *Lane 1.* Molecular weight standards. *Lane 2.* OMPs from the polymyxin-susceptible *K. pneumoniae* ATCC 13883. *Lane 3.* OMPs from the extremely polymyxin-resistant *K. pneumoniae* ATCC 13883. The OMPs profiling of both the polymyxin-susceptible and resistant strain of *K. pneumoniae* ATCC 13883 showed no difference in OmpK35 and OmpK36 bands.

Proteomic analysis identified 132 and 110 differentially represented OMPs in colistin-susceptible wild-type and the extremely colistin-resistant mutant, respectively, with the reference ATCC 13883 proteome (**Tables 5.1, 5.2, Supplementary Tables 5.3 and 5.4**). In

wild type and mutant, 122 (92.4%) and 103 (93.6%) OMPs have GO annotations based on the Uniprot database (**Supplementary Table 5.5**). Enrichment analysis revealed that 23 pathways were significantly overrepresented in wild type, including biological processes of carbohydrate metabolism, cell division, peptidoglycan biosynthesis and ATP hydrolysis coupled proton transport; whereas in the colistin-resistant mutant, 13 GO classes were overrepresented, including those associated with OMPs related to bacterial stress responses (**Supplementary Tables 5.6 and 5.7**).

In summary, 47 out of 132 (35.6%) sensitive strain OMPs and 38 out of 110 (34.5%) resistant strain OMPs were mapped onto metabolic pathways of type strain MGH78578, respectively (**Supplementary Tables 5.3-5.5**). Enrichment analysis showed that (i) Five metabolic pathways from BioCyc were overrepresented in the sensitive strain, including gluconeogenesis and pyruvate metabolism (**Figure 5.3A and Supplementary Table 5.8**); (ii) 17 metabolic pathways from BioCyc were overrepresented in the resistant strain, including glutamine degradation, pyruvate, aspartate and asparagine metabolism (**Figure 5.3B and Supplementary Table 5.9**).

Table 5.1 OMP proteome differences between paired colistin-susceptible and extremely colistin-resistant *K. pneumoniae* ATCC 13883 strains. OMPs are ranked in each column based on the number of peptides detected. The less abundant OMPs detected are listed in Table 5.2.

Sensitive *K. pneumoniae* ATCC 13883		**Resistant *K. pneumoniae* ATCC 13883**	
Protein name	**Number of peptides detected**	**Protein name**	**Number of peptides detected**
Outer membrane protein A precursor	37	Outer membrane protein A precursor	26
18K peptidoglycan-associated outer membrane lipoprotein; Peptidoglycan-associated lipoprotein precursor; Outer membrane protein P6; OmpA/MotB precursor	21	major outer membrane lipoprotein	18
major outer membrane lipoprotein	20	Pyruvate formate-lyase (EC 2.3.1.54)	17
Non-specific DNA-binding protein Dps / Iron-binding ferritin-like antioxidant protein / Ferroxidase (EC 1.16.3.1)	17	18K peptidoglycan-associated outer membrane lipoprotein; Peptidoglycan-associated lipoprotein precursor; Outer membrane protein P6; OmpA/MotB precursor	17
Glycerol kinase (EC 2.7.1.30)	16	Putative exported protein precursor	14
Translation elongation factor Tu	14	Translation elongation factor Tu	14
Outer membrane protein W	12	Outer membrane protein Imp, required for envelope biogenesis / Organic solvent tolerance protein precursor	12
Putative exported protein precursor	11	Non-specific DNA-binding protein Dps / Iron-binding ferritin-like antioxidant protein / Ferroxidase (EC 1.16.3.1)	12
Outer membrane protein Imp, required for envelope biogenesis / Organic solvent tolerance protein precursor	10	Glycerol kinase (EC 2.7.1.30)	12
Outer membrane protein X precursor	8	Outer membrane protein W precursor	9
Translation elongation factor G	8	Type I secretion outer membrane protein, TolC precursor	9
Nucleoside-specific channel-forming protein Tsx precursor	8	Transcriptional repressor of PutA and PutP / Proline dehydrogenase (EC 1.5.99.8) (Proline oxidase) / Delta-1-pyrroline-5-carboxylate dehydrogenase (EC 1.5.1.12)	8
Pyruvate formate-lyase (EC 2.3.1.54)	7	Enolase (EC 4.2.1.11)	7

NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)	7	Phosphoenolpyruvate synthase (EC 2.7.9.2)	7
Enolase (EC 4.2.1.11)	6	Outer membrane lipoprotein pcp precursor	6
NAD-specific glutamate dehydrogenase (EC 1.4.1.2); NADP-specific glutamate dehydrogenase (EC 1.4.1.4)	6	Translation elongation factor G	6
probable lipoprotein	5	NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)	6
SSU ribosomal protein S4p (S9e)	5	Nucleoside-specific channel-forming protein Tsx precursor	6
Cytochrome d ubiquinol oxidase subunit I (EC 1.10.3.-)	5	Cytochrome d ubiquinol oxidase subunit I (EC 1.10.3.-)	6
Outer membrane protein assembly factor YaeT precursor	5	probable lipoprotein	5
Acetate kinase (EC 2.7.2.1)	5	NAD-specific glutamate dehydrogenase (EC 1.4.1.2); NADP-specific glutamate dehydrogenase (EC 1.4.1.4)	5
Heat shock protein 60 family chaperone GroEL	4	Lysine decarboxylase, inducible (EC 4.1.1.18)	5
LSU ribosomal protein L2p (L8e)	4	Heat shock protein 60 family chaperone GroEL	5
Asparagine synthetase [glutamine-hydrolyzing] (EC 6.3.5.4)	4	Outer membrane protein X precursor	5
Transcription termination factor Rho	4	Phosphate acetyltransferase (EC 2.3.1.8)	4
SSU ribosomal protein S2p (SAe)	4	Glucosamine--fructose-6-phosphate aminotransferase [isomerizing] (EC 2.6.1.16)	4
Transcriptional repressor of PutA and PutP / Proline dehydrogenase (EC 1.5.99.8) (Proline oxidase) / Delta-1-pyrroline-5-carboxylate dehydrogenase (EC 1.5.1.12)	3	LSU ribosomal protein L2p (L8e)	4
Universal stress protein G	3	Outer membrane protein assembly factor YaeT precursor	4
Glucosamine--fructose-6-phosphate aminotransferase [isomerizing] (EC 2.6.1.16)	3	Fructose-bisphosphate aldolase class II (EC 4.1.2.13)	4

Uronate isomerase (EC 5.3.1.12)	3	Universal stress protein G	3
Outer membrane lipoprotein pcp precursor	3	PTS system, glucose-specific IIB component (EC 2.7.1.69) / PTS system, glucose-specific IIC component (EC 2.7.1.69)	3
Alcohol dehydrogenase (EC 1.1.1.1); Acetaldehyde dehydrogenase (EC 1.2.1.10)	3	Acetate kinase (EC 2.7.2.1)	3
Uncharacterized membrane protein YqjD	3	Transcription termination factor Rho	3
Phosphoenolpyruvate synthase (EC 2.7.9.2)	3	Thiamin-phosphate pyrophosphorylase (EC 2.5.1.3)	3
Type I secretion outer membrane protein, TolC precursor	3	SSU ribosomal protein S3p (S3e)	3
Myo-inositol 2-dehydrogenase 1 (EC 1.1.1.18)	2	Aspartate ammonia-lyase (EC 4.3.1.1)	2
Isocitrate dehydrogenase [NADP] (EC 1.1.1.42)	2	LPS-assembly lipoprotein RlpB precursor (Rare lipoprotein B)	2
Nucleoside permease NupC	2	Alcohol dehydrogenase (EC 1.1.1.1); Acetaldehyde dehydrogenase (EC 1.2.1.10)	2
Hydroxymethylpyrimidine phosphate kinase ThiD (EC 2.7.4.7)	2	PTS system, mannose-specific IIA component (EC 2.7.1.69) / PTS system, mannose-specific IIB component (EC 2.7.1.69)	2
Alpha-galactosidase (EC 3.2.1.22)	2	GMP synthase [glutamine-hydrolyzing] (EC 6.3.5.2)	2
Universal stress protein E	2	Uncharacterized membrane protein YqjD	2
ATP synthase beta chain (EC 3.6.3.14)	2	PTS system, N-acetylglucosamine-specific IIA component (EC 2.7.1.69) / PTS system, N-acetylglucosamine-specific IIB component (EC 2.7.1.69) / PTS system, N-acetylglucosamine-specific IIC component (EC 2.7.1.69)	2
SSU ribosomal protein S3p (S3e)	2	Alkyl hydroperoxide reductase protein C (EC 1.6.4.-)	2
PTS system, glucose-specific IIB component (EC 2.7.1.69) / PTS system, glucose-specific IIC component (EC 2.7.1.69)	2	Hydroxymethylpyrimidine phosphate kinase ThiD (EC 2.7.4.7)	2
Lipoprotein	2	Pyruvate formate-lyase (EC 2.3.1.54)	2
2-oxoglutarate dehydrogenase E1 component (EC 1.2.4.2)	2	Osmotically inducible lipoprotein E precursor	2
SSU ribosomal protein S1p	2	SSU ribosomal protein S7p (S5e)	2

Putative lipoprotein	2	SSU ribosomal protein S4p (S9e)	2
Adenylosuccinate synthetase (EC 6.3.4.4)	2	ATP synthase alpha chain (EC 3.6.3.14)	2
LSU ribosomal protein L10p (P0)	2	Alkyl hydroperoxide reductase subunit C-like protein	2
Aspartate ammonia-lyase (EC 4.3.1.1)	2	Glutamine synthetase type I (EC 6.3.1.2)	2
SSU ribosomal protein S7p (S5e)	2	Hexuronate transporter	2
Fructose-bisphosphate aldolase class II (EC 4.1.2.13)	2	S-adenosylmethionine synthetase (EC 2.5.1.6)	2
Cytochrome d ubiquinol oxidase subunit II (EC 1.10.3.-)	2	Phosphoenolpyruvate carboxykinase [ATP] (EC 4.1.1.49)	1
Signal recognition particle, subunit Ffh SRP54 (TC 3.A.5.1.1)	2	Maltose/maltodextrin transport ATP-binding protein MalK (EC 3.6.3.19)	1

Table 5.2 OMP proteome differences between paired colistin-susceptible and extremely colistin-resistant *K. pneumoniae* ATCC 13883 strains.

Sensitive *K. pneumoniae* ATCC 13883		**Resistant *K. pneumoniae* ATCC 13883**	
Protein name	**Number of peptides detected**	**Protein name**	**Number of peptides detected**
Cobyric acid synthase	1	4-aminobutyraldehyde dehydrogenase (EC 1.2.1.19)	1
Phosphoheptose isomerase 1 (EC 5.3.1.-)	1	Galactose/methyl galactoside ABC transport system, permease protein MglC (TC 3.A.1.2.3)	1
Purine nucleoside phosphorylase (EC 2.4.2.1)	1	LSU ribosomal protein L3p (L3e)	1
FIG004088: inner membrane protein YebE	1	Galactose/methyl galactoside ABC transport system, ATP-binding protein MglA (EC 3.6.3.17)	1

Selenocysteine-specific translation elongation factor	1	SSU ribosomal protein S2p (SAe)	1
Sulfur carrier protein adenylyltransferase ThiF	1	Myo-inositol 2-dehydrogenase 1 (EC 1.1.1.18)	1
Maltose/maltodextrin transport ATP-binding protein MalK (EC 3.6.3.19)	1	Glutathione S-transferase (EC 2.5.1.18)	1
ATP-dependent protease La (EC 3.4.21.53) Type I	1	LSU ribosomal protein L5p (L11e)	1
Glycoprotein-polysaccharide metabolism	1	Threonyl-tRNA synthetase (EC 6.1.1.3)	1
DNA-binding protein H-NS	1	Purine nucleoside phosphorylase (EC 2.4.2.1)	1
PTS system, mannose-specific IIA component (EC 2.7.1.69) / PTS system, mannose-specific IIB component (EC 2.7.1.69)	1	Osmotically inducible lipoprotein B precursor	1
Glycerol-3-phosphate acyltransferase (EC 2.3.1.15)	1	Nucleoside permease NupC	1
LPS-assembly lipoprotein RlpB precursor (Rare lipoprotein B)	1	NADH-ubiquinone oxidoreductase chain M (EC 1.6.5.3)	1
Cell division protein FtsA	1	LSU ribosomal protein L13p (L13Ae)	1
Adenylate cyclase (EC 4.6.1.1)	1	Serine hydroxymethyltransferase (EC 2.1.2.1)	1
PTS system, N-acetylglucosamine-specific IIA component (EC 2.7.1.69) / PTS system, N-acetylglucosamine-specific IIB component (EC 2.7.1.69) / PTS system, N-acetylglucosamine-specific IIC component (EC 2.7.1.69)	1	Phage shock protein A	1
Hypothetical lipoprotein YajG precursor	1	FIG00638953: hypothetical protein	1
Small heat shock protein	1	Alcohol dehydrogenase (EC 1.1.1.1)	1
Lysyl-tRNA synthetase (class II) (EC 6.1.1.6)	1	Sulfur carrier protein adenylyltransferase ThiF	1
Putative outer membrane protein	1	LSU ribosomal protein L22p (L17e)	1
Putative phosphosugar isomerase	1	Putative lipoprotein	1
Cysteine synthase (EC 2.5.1.47)	1	21 kDa hemolysin precursor	1
Threonyl-tRNA synthetase (EC 6.1.1.3)	1	Glutamine ABC transporter, periplasmic glutamine-binding protein (TC 3.A.1.3.2)	1
Transcriptional activator of maltose regulon, MalT	1	Universal stress protein E	1

4-aminobutyraldehyde dehydrogenase (EC 1.2.1.19)	1	Tail-specific protease precursor (EC 3.4.21.102)	1
Acetaldehyde dehydrogenase, ethanolamine utilization cluster	1	FIG00732539: hypothetical protein	1
Chromosome partition protein MukB	1	Maltoporin (maltose/maltodextrin high-affinity receptor, phage lambda receptor protein)	1
UDP-N-acetylmuramoylalanyl-D-glutamate--2,6-diaminopimelate ligase (EC 6.3.2.13)	1	SSU ribosomal protein S1p	1
Osmotically inducible lipoprotein B precursor	1	6-phosphofructokinase class II (EC 2.7.1.11)	1
ATP synthase A chain (EC 3.6.3.14)	1	LSU ribosomal protein L15p (L27Ae)	1
Alkyl hydroperoxide reductase protein C (EC 1.6.4.-)	1	Cobalt-precorrin-2 C20-methyltransferase (EC 2.1.1.130)	1
Pyruvate formate-lyase (EC 2.3.1.54)	1	Lipoprotein	1
Galactose/methyl galactoside ABC transport system, permease protein MglC (TC 3.A.1.2.3)	1	LSU ribosomal protein L1p (L10Ae)	1
Cell division protein FtsZ (EC 3.4.24.-)	1	SSU ribosomal protein S5p (S2e)	1
LSU ribosomal protein L24p (L26e)	1	Pyruvate dehydrogenase E1 component (EC 1.2.4.1)	1
S-adenosylmethionine synthetase (EC 2.5.1.6)	1	Entericidin B precursor	1
Inosine-5'-monophosphate dehydrogenase (EC 1.1.1.205)	1	Maltose/maltodextrin ABC transporter, substrate binding periplasmic protein MalE	1
Phosphoglycerate kinase (EC 2.7.2.3)	1	Asparagine synthetase [glutamine-hydrolyzing] (EC 6.3.5.4)	1
LSU ribosomal protein L22p (L17e)	1	Putative phosphosugar isomerase	1
LSU ribosomal protein L3p (L3e)	1	Chaperone protein HtpG	1
SgrR, sugar-phosphate stress, transcriptional activator of SgrS small RNA	1	LSU ribosomal protein L20p	1
LSU ribosomal protein L6p (L9e)	1	Cysteine synthase (EC 2.5.1.47)	1
GTP-binding protein TypA/BipA	1	Cytochrome d ubiquinol oxidase subunit II (EC 1.10.3.-)	1
LSU ribosomal protein L5p (L11e)	1	Small heat shock protein	1
DNA-directed RNA polymerase beta' subunit (EC 2.7.7.6)	1	Aspartate--ammonia ligase (EC 6.3.1.1)	1
Adenosine deaminase (EC 3.5.4.4)	1	Membrane fusion protein of RND family multidrug efflux pump	1

NADP-dependent malic enzyme (EC 1.1.1.40)	1	Septum site-determining protein MinD	1
IncF plasmid conjugative transfer surface exclusion protein TraT	1	Chaperone protein DnaK	1
LSU ribosomal protein L28p	1	LSU ribosomal protein L10p (P0)	1
Entericidin B precursor	1	Di/tripeptide permease DtpB	1
UDP-N-acetylglucosamine 1-carboxyvinyltransferase (EC 2.5.1.7)	1	Carbamoyl-phosphate synthase large chain (EC 6.3.5.5)	1
LSU ribosomal protein L1p (L10Ae)	1	ABC transporter, ATP-binding protein	1
Lysine decarboxylase, inducible (EC 4.1.1.18)	1	Uncharacterized protein YeaG	1
Formate efflux transporter (TC 2.A.44 family)	1	Uridine phosphorylase (EC 2.4.2.3)	1
Maltose-6'-phosphate glucosidase (EC 3.2.1.122)	1	5'-methylthioadenosine nucleosidase (EC 3.2.2.16) / S-adenosylhomocysteine nucleosidase (EC 3.2.2.9)	1
Hexuronate transporter	1		
Preprotein translocase secY subunit (TC 3.A.5.1.1)	1		
Septum site-determining protein MinD	1		
Maltoporin (maltose/maltodextrin high-affinity receptor, phage lambda receptor protein)	1		
SSU ribosomal protein S18p @ SSU ribosomal protein S18p, zinc-independent	1		
Phosphate acetyltransferase (EC 2.3.1.8)	1		
Osmotically inducible lipoprotein E precursor	1		
LSU ribosomal protein L21p	1		
Sugar diacid utilization regulator SdaR	1		
PTS system, sucrose-specific IIB component (EC 2.7.1.69) / PTS system, sucrose-specific IIC component (EC 2.7.1.69)	1		
Uridine phosphorylase (EC 2.4.2.3)	1		
Alkyl hydroperoxide reductase subunit C-like protein	1		
Translation initiation factor 2	1		

Rod shape-determining protein MreB	1	
UDP-N-acetylglucosamine--N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase (EC 2.4.1.227)	1	
SSU ribosomal protein S5p (S2e)	1	
LSU ribosomal protein L9p	1	
5-keto-2-deoxygluconokinase (EC 2.7.1.92) / uncharacterized domain	1	
Aspartate aminotransferase (EC 2.6.1.1)	1	
Glucose-6-phosphate isomerase (EC 5.3.1.9)	1	
ATP synthase alpha chain (EC 3.6.3.14)	1	
FIG00732100: hypothetical protein	1	

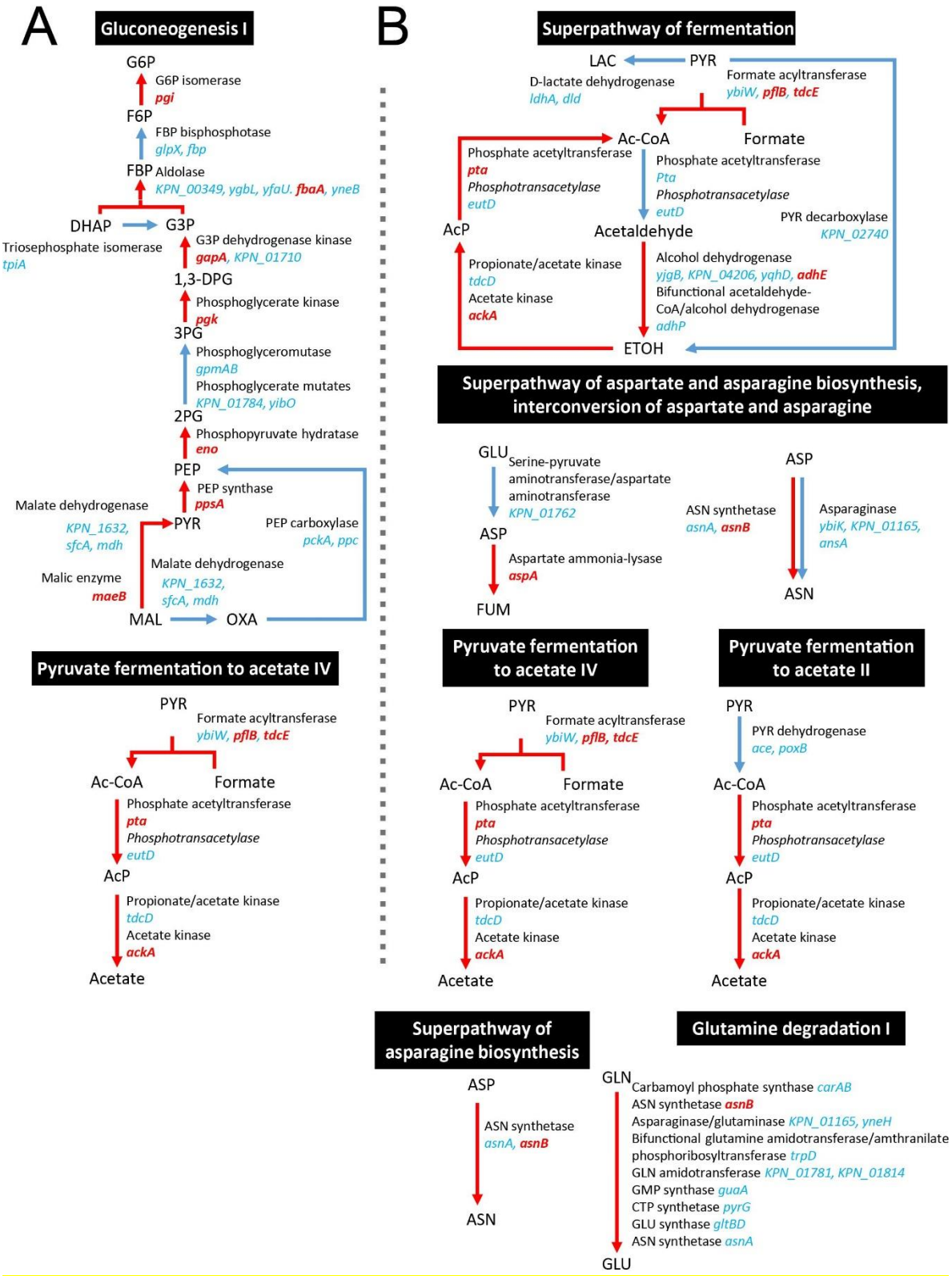

Figure 5.3 Mapping of identified outer membrane proteins of the (**A**) colistin-susceptible *K. pneumoniae* ATCC 13883 and (**B**) the paired extremely colistin-resistant *K. pneumoniae* ATCC 13883

on to the metabolic pathways of MGH 78578 from BioCyc. Abbreviations: G6P, β-D-glucose 6-phosphate; F6P, fructose 6-phosphate; FBP, fructose 1,6-biphosphate; DHAP, dihydroxyacetone 3-phosphate; G3P, glyceraldehyde-3-phosphate; 1,3-DPG, 1 3-bisphosphoglycerate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; PYR, pyruvate; MAL, (*S*)-malate; OXA, oxaloacetate; Ac-CoA, acetyl-CoA; AcP, acetylphosphate; LAC, (*R*)-lactate; ETOH, ethanol; GLU, L-glutamate; GLN, L-glutamine; ASP, L-aspartate; ASN, L-asparagine; FUM, fumarate. (Figure was generated in BioCyc).

5.3.2 Genetic mutations associated with high-level polymyxin resistance in *K. pneumoniae* ATCC 13883

Polymyxin resistance in *K. pneumoniae* involves the multi-tier up-regulation of capsular polysaccharide expression, and the systems required for the modification of lipid A with 4-amino-4-deoxy-L-arabinose (173, 429, 432, 434-440). In *K. pneumoniae* the expression of 4-amino-4-deoxy-L-arabinose modifications to the lipid A phosphates is under the control of the two component regulatory systems [PhoPQ-PmrD]-PmrAB that are activated in response to low pH, low magnesium and high iron (424, 432). Specifically, the PhoP-PhoQ (collectively PhoPQ) system regulates the magnesium regulon which may activate polymyxin B resistance genes (*pmrHFIJKLM*) encoding biosynthesis and lipid A transfer of 4-amino-4-deoxy-L-arabinose under low magnesium conditions (196). This PhoP-PhoQ system is also connected by the connector proteins PmrD and MgrB which act as feed-back modulators between the PhoPQ and PmrAb (196). PhoPQ regulates the activation of PmrD which can then bind to PmrA and prolong its phosphorylation state, eventually activating the expression of the PmrA-PrmB (collectively PmrAB) system to promote resistance to polymyxin (196). MgrB is the negative regulator of PhoQ, its inactivation due to the insertion elements or frame-shift mutations that lead to premature inactivation (commonly seen in polymyxin-resistant clinical

isolates) results in the overexpression of PhoPQ and lipid A modification (196, 424, 501-505). Mutations in PmrAB and PhoPQ have also been reported in polymyxin-resistant clinical isolates of *K. pneumoniae* (196, 424, 501-505). In addition to the aforementioned *mgrB* inactivation and dysregulation of the two-component systems (TCS), we have previously reported that the under-acylation of lipid A increases the polymyxin susceptibility of *K. pneumoniae;* which highlights that the decoration of lipid A with additional fatty acyl chains is important for polymyxin resistance (441, 442, 506). Herein, we sequenced the genome of both the colistin-susceptible and -resistant *K. pneumoniae* ATCC 13883 strains and performed a comparative scan of their genomes. Comparative genomic analysis revealed that the *mgrB* gene of the colistin-resistant strain is inactivated by a single nucleotide insertion at position 40 (39_40insT) resulting in a frame-shift and premature termination (A15SfsX25) (**Figure 5.4**). Furthermore, we detected four synonymous mutations *phoP* (363T>C and 664T>C) *and phoQ* (309A>C and 1185C>T) (**Figures 5.5 & 5.6**) (196, 507).

mgrB

Query: *mgrB* from *K. pneumoniae* ATCC 13883
Subject: COL100 genome
Results: an insertion at position 40 (c.39_40insT), resulting in a frame shift and a premature fragment (p.A15SfsX25)

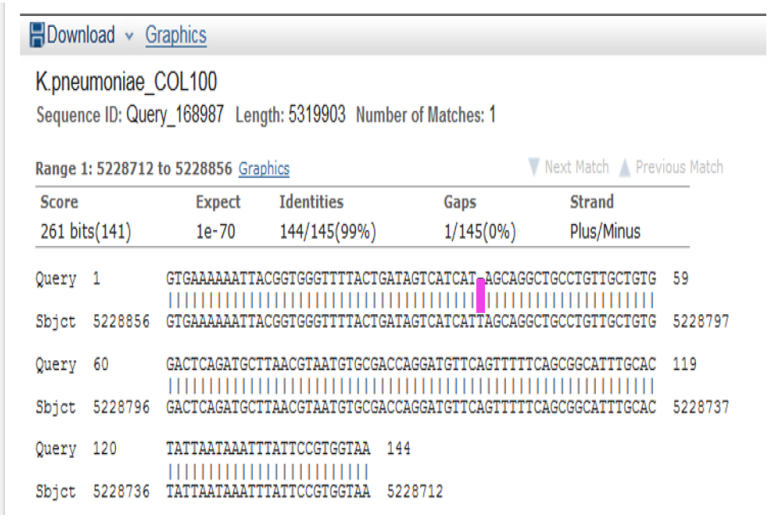

Figure 5.4 Alignment of the *mgrB* genes of the colistin-susceptible *K. pneumoniae* ATCC 13883 (GenBank: JSZI01000008.1) and the paired extremely colistin-resistant *K. pneumoniae* ATCC 13883.

PhoP

Query: *PhoP* from *K. pneumoniae* ATCC 13883
Subject: COL100 genome
Results: two synonymous mutations (c.363T>C and c.664T>C)

- **Virulence transcriptional regulatory protein PhoP**
  lcl|JSZI01000005.1_cds_KHF67519.1_273 [gene=phoP_1] [locus_tag=LT32_00274] [protein=Virulence transcriptional regulatory protein PhoP] [protein_id=KHF67519.1] [location=38237..38908] [gbkey=CDS]

Figure 5.5 Alignment of the *PhoP* genes of the colistin-susceptible *K. pneumoniae* ATCC 13883 (GenBank: JSZI01000008.1) and the paired extremely colistin-resistant *K. pneumoniae* ATCC 13883.

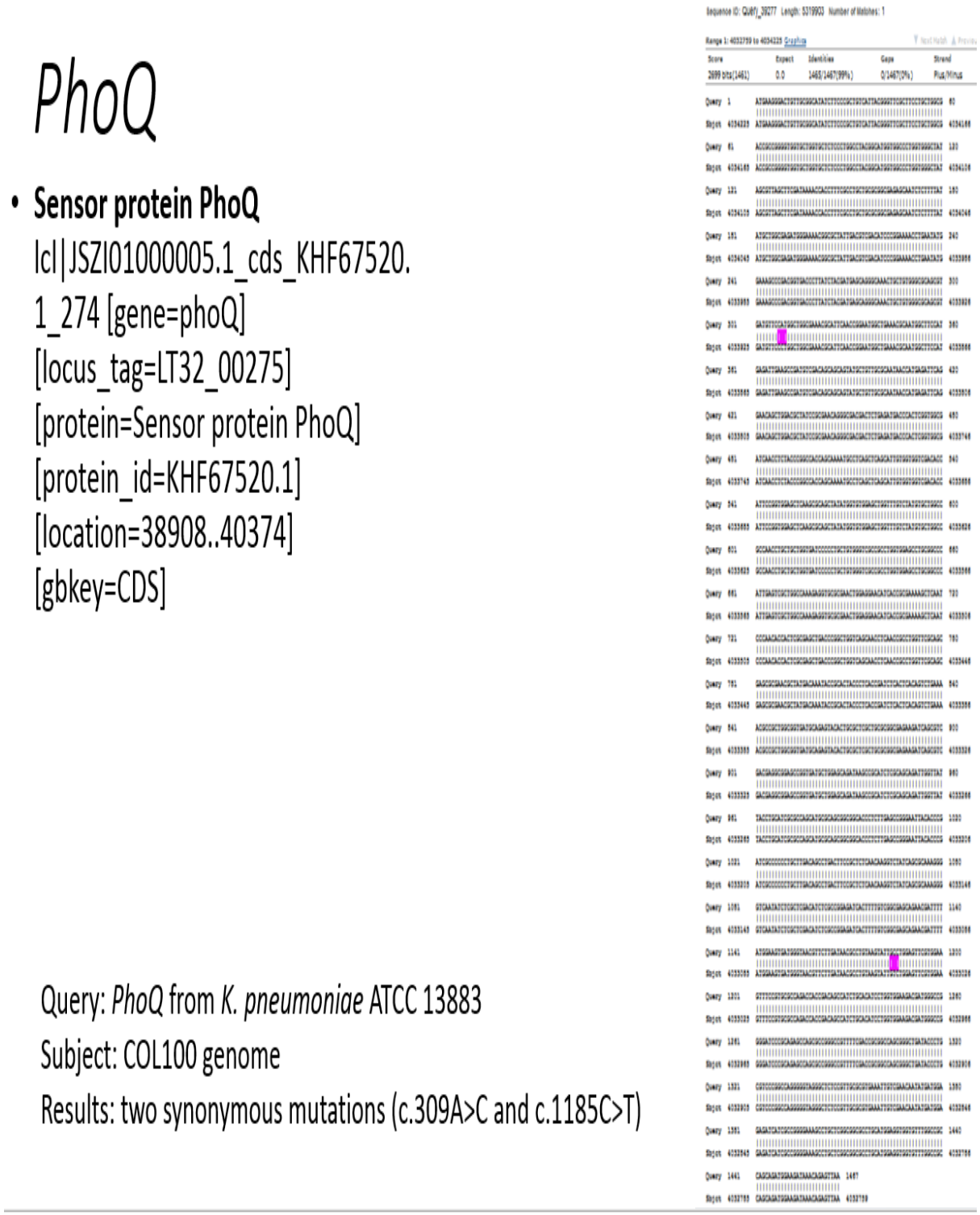

Figure 5.6 Alignment of the *PhoQ* genes of the colistin-susceptible *K. pneumoniae* ATCC 13883 (GenBank: JSZI01000008.1) and the paired extremely colistin-resistant *K. pneumoniae* ATCC 13883.

5.3.3 Lipid A remodelling

The consensus structure of lipid A is represented by a β-1′-6-linked D-glucosamine disaccharide that is phosphorylated at the 1- and 4′-positions (436). For *K. pneumoniae*, this backbone is attached to six or seven acyl chains, (*R*)-3-hydroxymyristoyl chains are attached to positions 2, 2′, 3 and 3′ of the glucosamine disaccharide, while secondary myristoyl chains are attached to the (*R*)-3-hydroxymyristoyl groups at the 2′ and 3′ positions (2′-3-OH-C_{14} and 3′-3-OH-C_{14}) and a secondary palmitoyl group attached to the (*R*)-3-hydroxymyristoyl group at the 2 position (2-3-OH-C_{16}) (**Figure 5.7A**) (441, 508, 509). Additionally, secondary palmitoylation has been observed at position 2 (2-3-OH-C_{16}) in a minor population of wild-type *K. pneumoniae* strains (441). In the mass spectrum of lipid A isolated from the polymyxin-susceptible *K. pneumoniae* ATCC 13883 strain, we can see the two typical *hexa*-acylated lipid A species at *m/z* 1826 and 1843, and their dephosphorylated forms at m/z 1746 and 1763, respectively (**Figure 5.7A**). The two peaks at *m/z* 1985 and 2065 correspond to the *hepta*-acylated lipid A with a palmitoyl C_{16} secondary fatty acyl chain. A *tetra*-acylated lipid A species at m/z 1377 is also detected, the peak at *m/z* 1393 is possibly due to one OH adduct (+16). The mass spectrum of lipid A isolated the from the *K. pneumoniae* ATCC 13883 colistin-resistant strain also displayed major peaks at *m/z* 1377, 1746, 1763, 1826, 1843, and 2065 corresponding to the aforementioned lipid A species observed in the susceptible strain. The major difference is a peak at *m/z* 1956.85 which is due to a *hexa*-acylated lipid A species *m/z* 1825.68 with the addition of one 4-amino-4-deoxy-L-arabinose moiety (+131) (**Figure 5.7B**).

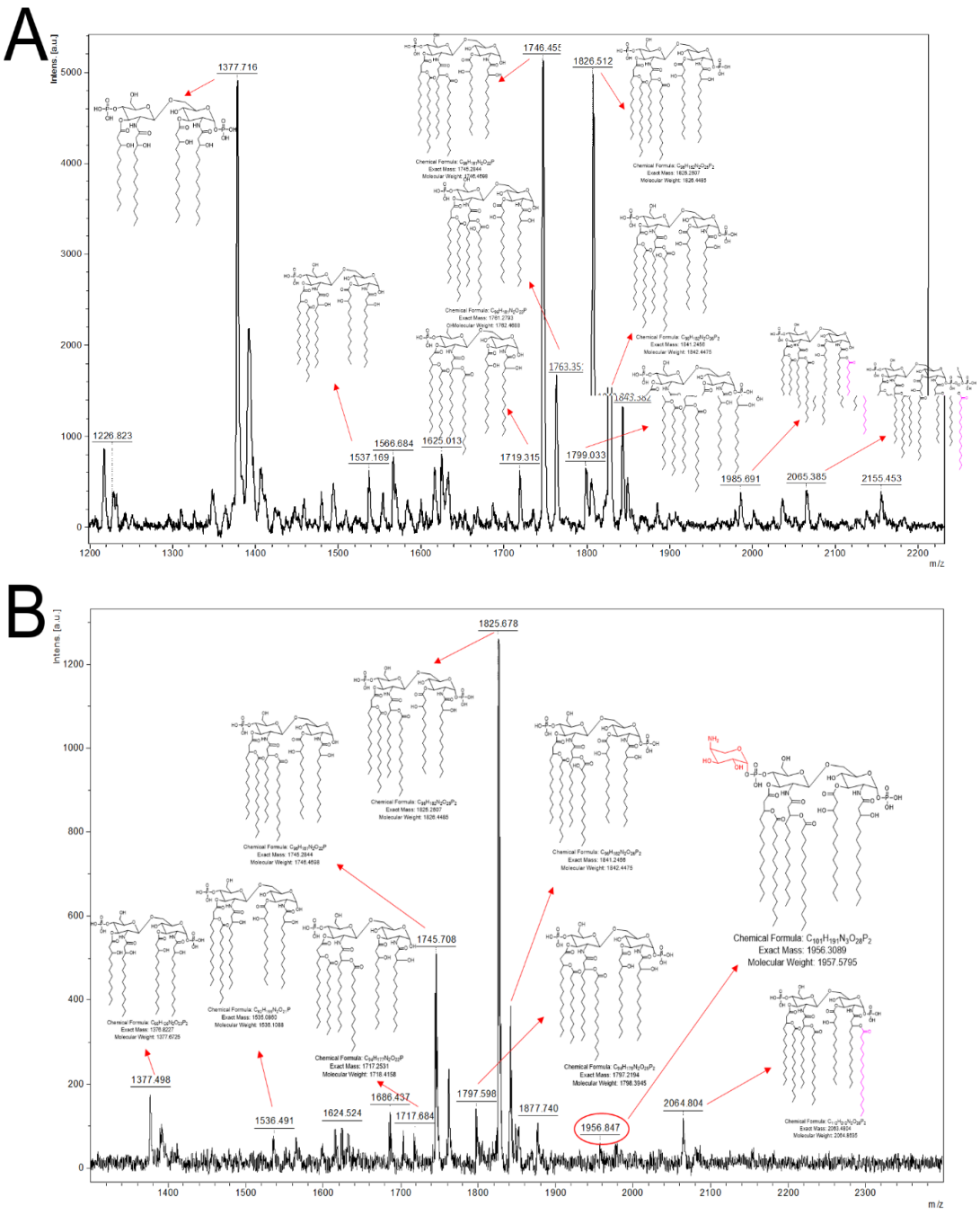

Figure 5.7 Lipid A profiles of (**A**) colistin-susceptible *K. pneumoniae* ATCC 13883 and (**B**) the paired extremely colistin-resistant *K. pneumoniae* ATCC 13883.

5.4 Conclusions

The present study is the first to reveal that the rubic for when a colistin-susceptible *K. pneumoniae* strain evolves into an extremely colistin-resistant strain subsumes several key

OMP and lipid A perturbations. In addition to the essential 4-amino-4-deoxy-L-arabinose modifications of lipid A due to inactivation of the *mgrB* TCS negative regulator; our proteomics analysis reveals that OMPs from bacterial stress response, glutamine degradation, pyruvate, aspartate and asparagine metabolic pathways were over represented in the OM proteome of the extremely colistin-resistant *K. pneumoniae* ATCC 13883 strain.

5.5 Supplementary tables

All supplementary tables are available online on the following link:

https://tinyurl.com/ybpw73zb

Table 5.3 Protein list in *Klebsiella pneumoniae* ATCC 13883 polymyxin-susceptable (KPS).

Table 5.3 Protein list in Klebsiella pneumor

Table 5.4 Protein list in Klebsiella pneumoniae ATCC 13883 polymyxin-resistantble (KPR).

Table 5.4 Protein list in Klebsiella pneumor

Table 5.5 Protein annotation of MGH78578 in UniProt.

Table 5.5 Protein annotation of MGH78

Table 5.6 Enriched GO terms in KPS.

Table 5.6 Enriched GO terms in KPS.xls

Table 5.7 Enriched GO terms in KPR.

Table 5.7 Enriched GO terms in KPR.xls

Table 5.8 Enriched BioCyc metabolic pathways in KPS.

Table 5.8 Enriched BioCyc metabolic path

Table 5.9 Enriched BioCyc metabolic pathways in KPR.

Table 5.9 Enriched BioCyc metabolic path

Chapter 6: Conclusions and Future Directions

6.1 Conclusions

MDR Gram-negative bacteria, in particular *P. aeruginosa, A. baumannii* and *K. pneumoniae* represent a serious global health crisis due to their ability to develop resistance to all clinically available antibiotics. The CDC has classified these pathogens as a "serious threat" (12). In addition, in 2017 WHO has classified them as Critical at the top of the Priority Pathogens List (13). With the dry antibiotic development pipeline and dose-associated toxicities for many antibiotics such as polymyxins and daptomycin, antibiotic combination therapy has become the preferred option for combating MDR Gram-negative pathogens and minimising both the emergence of resistance and toxicity (510). This is of particular interest when the combination includes the last resort antimicrobial compounds such as polymyxins (208). The empirical use of antibiotic combinations does not necessarily provide complete eradication of these pathogens and improve the clinical outcome. Therefore, the real obstacle is how to optimise clinically useful antimicrobial combinations, and this necessitates a comprehensive understanding of mechanisms of action bacterial resistance. In general, antibiotics are classified as either bactericidal or bacteriostatic, and interfering with the cell wall, DNA, RNA or protein synthesis (511). However, the effect of antibiotics on bacterial metabolism at the cellular network level remains mostly ambiguous. Omics studies including proteomics and lipidomics provides a powerful tool for better understanding the antibiotic-pathogen interactions at the cellular level (512, 513). This PhD thesis aimed not only to identify optimal synergistic antimicrobial combinations but also to elucidate the antibiotic-pathogen interactions through integrating proteomics and lipidomics studies.

In **Chapter 2** of this PhD project, the activity of the novel combination of polymyxin B with silver NPs was *in vitro* assessed using the checkerboard assay against a wide range of polymyxin-susceptible and –resistant Gram-negative isolates, in particular polymyxin-resistant *P. aeruginosa* CF, *A. baumannii* and *K. pneumoniae* isolates. The combination of polymyxin B and silver NPs was synergistic against most of the tested pathogens, in particular, against the problematic polymyxin-resistant *P. aeruginosa* CF isolates. The synergistic polymyxin B-silver NPs combination was further assessed by statistic kill studies against polymyxin-resistant non-mucoid *P. aeruginosa* (FADDI-PA066 and FADDI-PA067) CF isolates, one polymyxin B resistant *A. baumannii* FADDI-AB143 isolate and one polymyxin B resistant *K. pneumoniae* FADDI-KP027. Notably, the combination was synergistic against the four tested isolates. Mechanistic studies of the synergytic killing effect included, nitrocefin, ROS production and ASM assays against polymyxin-resistant non-mucoid *P. aeruginosa* (FADDI-PA066) CF isolates revealed that the combination was more effective in producing OM damage than either compound *per se*. Moreover, confocal and electron microscopy imaging provided further evidence about the OM destructive effect of the combination. Concentrations of both polymyxin B and silver NPs used in this study represented clinically achievable concentrations. Overall, this study lays the foundation for the development of novel polymyxin B-silver NPs combination to treat life-threatening MDR lung infections in CF patients. Realization of this objective will require lung infection models and PK/PD studies (*cf.* **Future Directions**).

Even with the combination of polymyxins with other antibiotics including silver NPs, still, there are some MDR gram-negative bacteria resistant to the treatment. Therefore, to garner clearer insights into the polymyxins-pathogen interactions, comparative lipidomics and proteomics studies were performed **chapters 3 and 4** to investigate the effect of polymyxins treatment on the selective sorting of proteins and lipids from both polymyxins-susceptible and

-resistant *K. pneumoniae* isolates into OMVs sub-proteome and -lipidome. In **Chapter 3**, the effect of polymyxin B (2 mg/L) treatment on sub-proteome of OMVs isolated from polymyxin-susceptible and -resistant *K. pneumoniae* ATCC700721 isolates was comparatively studied. Treatment with polymyxin B significantly affected the protein cargo of OMVs isolated from pre-treated polymyxin-susceptible and –resistant *K. pneumoniae* ATCC700721 isolates. Moreover, pathway analysis revealed that the expression of key proteins involved in Aminoacyl-tRNA biosynthesis, Lipopolysaccharide biosynthesis and peptidoglycan biosynthesis pathways were significantly overrepresented in response to the polymyxin B treatment, particularly in the OMVs of the susceptible isolates. Whereas, polymyxin B treatment of both susceptible and resistant strains resulted in significant downregulation of ABC transporters, protein export, two-component system, cationic antimicrobial peptide (CAMP) resistance and beta-lactam resistance proteins. The most significantly reduced CAMP resistance proteins were those involved in polymyxins resistance, namely the sensor histidine kinase PhoQ (involved in PhoQ-PhoP two-component regulatory system), 4-amino-4-deoxy-L-arabinose transferase (mediates lipid A modification) and cationic peptide transport system substrate-binding protein. Although there is a complexity in selective packaging of OMVs sub-proteome from both susceptible and resistant strains, however, the finding from this study paves the way toward better understanding of the role of OMVs in bacterial defence against membrane-targeting antibiotics. C**hapter 4** evaluated the effect of polymyxin B treatment on the sub-lipidome of OMVs isolated from two clinical pairs of polymyxin-susceptible and -resistant *K. pneumoniae* isolates (*K. pneumoniae* FADDI-KP069 and *K. pneumonia* BM3) and one laboratory pair of polymyxin-susceptible and -resistant *K. pneumoniae* (*K. pneumoniae* ATCC700721). Initially, DLS and TEM analysis showed that the particle size of the OMVs isolated from polymyxin B treated strains was larger than that of the untreated control samples.

Moreover, the treated resistant strain secreted OMVs with a larger particle size compared to the treated paired susceptible strain. In addition, the comparative lipidomics analysis demonstrated that polymyxin B treatment of polymyxin-susceptible isolates significantly reduced the production of glycerophospholipids, fatty acyls and sphingolipids in the OMVs; conversely, the lipid content of OMVs from resistant strains significantly increased in response to treatment with polymyxin B. The current study supports the notion that polymyxin-resistant *K. pneumoniae* attempts to increase the rigidity of its envelope upon exposure to polymyxin B; and the role of OMVs in bacterial resistant to cationic antimicrobial compounds. Overall, this finding further supports our pervious study **(chapter 3)**.

Chapter 5 performed a comparative bioinformatic and proteomic analyses for the OM proteome and lipid A profiles of paired colistin-susceptible and extremely colistin-resistant *K. pneumoniae* ATCC 13883 strains. Lipid A profiling revealed that the OM of the colistin-susceptible strain is decorated primarily by hexa- and hepta acylated lipid A species and a minor tetra-acylated species. While, In the lipid A profile of the extremely colistin-resistant strain, in addition to the aforementioned lipid A species, modification of the hexa-acylated lipid with the 4-amino-4-deoxy-L-arabinose A was detected. Our group previously reported that decoration of lipid A with additional fatty acyl chains increases the resistance of *K. pneumoniae* to polymyxins. Comparative profiling of the OM proteome of each strain revealed that OMPs from bacterial stress response, glutamine degradation, pyruvate, aspartate and asparagine metabolic pathways were over represented in the extremely colistin-resistant *K. pneumoniae* ATCC 13883 strain. However, in the sensitive strain, OMPs from carbohydrate metabolism, H+-ATPase, cell division and peptidoglycan biosynthesis were over represented. OM protein profiling showed no difference in OmpK35 and OmpK36 between polymyxin-

susceptible and resistant strain of *K. pneumoniae* ATCC 13883. This finding suggests that the deficiency of these porin proteins might not involve in resistance of these strains to polymyxins. In conclusion, this thesis has identified a novel combination of polymyxin B-silver NPs, which could be a viable option for fighting MDR-Gram-negative pathogens, including MDR polymyxin-resistant *P. aeruginosa* associated with CF lung infection. The combination improved the antimicrobial activity of polymyxin B against polymyxin-resistant CF isolates. The enhanced antimicrobial activity of the combination might involve their combined membrane-damaging activity, which was confirmed by TEM and confocal imaging. Furthermore, the findings from omics studies revealed that there are several important pathways/genes in *K. pneumoniae* with relation to bacterial virulence and polymyxins resistance. Overall, the data reported in this thesis shed light on useful strategies to rescue the clinical effectiveness of the last-line therapy (polymyxins) against Gram-negative 'superbugs'.

6.2 Future Directions

The *in vitro* antibacterial synergistic activity of polymyxin B-silver NPs in **chapter 1** was investigated against a wide range of Gram-negative bacteria, particularly against MDR polymyxin-resistant *P. aeruginosa* isolates from the CF lung. Further studies involve lung infection models with these isolates are warrented to evaluate there *in vivo* efficacy of the combination. Moreover, *in vitro* as well as *in vivo* PK/PD models are essential for further optimisation of the combination dosage regimens. In addition, omics studies including genomics and transcriptomics are warranted to elucidate the mechanism(s) of synergy.

The untargeted lipidomics and proteomics studies in **chapter 3** and **4** demonstrated the effect of polymyxin B on the proteome and lipidome of OMVs isolated from of polymyxin-susceptible and -resistant *K. pneumoniae* isolates; and identified key protein and lipid species

that are selectively packaged from the parent bacteria into the OMV sub-lipidome and –proteome. In addition, the OM proteome and lipid A profiling in **chapter 5** highlights the OM remodelling associated with extremely high levels of polymyxins resistance in *K. pneumoniae*. However, these studies should be followed up by targeted lipidomics and proteomics studies to pinpoint the pathways/genes that are specifically involved in polymyxins mechanisms of action and resistance. Moreover, it is of a great importance to perform a proteomic and lipidomic analysis of the OM and OMVs derived from other problematic pathogens (*P. aeruginosa A. baumannii*) and compare the result with that obtained in **chapters 3, 4** and **5** to demonstrate the difference in response to polymyxins between all the species. Furthermore, comparative analysis of the proteome and lipidome of the whole bacterial cell for *P. aeruginosa A. baumannii* and *K. pneumoniae*, in particular the clinical isolates, is warranted, which could provide a clear insight about the selective sorting of the proteins and lipids into the OMVs.

In summary, as polymyxins are important last –line antibiotics against MDR Gram-negative 'superbugs', research on optimising their use is of great importance. To the best of our knowledge, this thesis is the first to identify a synergistic polymyxin combination with silve-NPs against MDR polymyxin-resistant *P. aeruginosa* isolated from CF lung infections. This thesis is also the first to demonstrate the effect of polymyxin B on lipidome and proteome of OMVs isolated from polymyxin-susceptible and -resistant *K. pneumoniae* isolates and perform a comparative bioinformatic and proteomic analyses for the OM proteome and lipid A profiles of paired colistin-susceptible and extremely colistin-resistant *K. pneumoniae* ATCC 13883 strains. Taken together, this thesis has significantly contributed to better understanding the polymyxins-pathogens interactions to combat life-threatening infections caused by MDR Gram-negative bacteria in the era of '*Bad Bugs, No Drugs*'.

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8 Additional Academic Contribution during PhD candidature

8.1 Appendix 1

Hussein MH, Schneider EK, Elliott A., Han M, Reyes-Ortega F, Morris F, Blastovich M, ***Jasim R****, Currie B, Mayo M, Baker M, Cooper MA, Li J and Velkov T:* **From breast cancer to antimicrobial: Combating extremely-resistant Gram-negative 'superbugs' using novel combinations of polymyxin B with selective estrogen receptor modulators**, Microb Drug Resist. 2017 Jul;23(5):640-650. doi: 10.1089/mdr.2016.0196. Epub 2016 Dec 9.

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From Breast Cancer to Antimicrobial: Combating Extremely Resistant Gram-Negative "Superbugs" Using Novel Combinations of Polymyxin B with Selective Estrogen Receptor Modulators

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Novel therapeutic approaches are urgently needed to combat nosocomial infections caused by extremely drug-resistant (XDR) "superbugs." This study aimed to investigate the synergistic antibacterial activity of polymyxin B in combination with selective estrogen receptor modulators (SERMs) against problematic Gram-negative pathogens. *In vitro* synergistic antibacterial activity of polymyxin B and the SERMs tamoxifen, raloxifene, and toremifene was assessed using the microdilution checkerboard and static time–kill assays against a panel of Gram-negative isolates. Polymyxin B and the SERMs were ineffective when used as monotherapy against polymyxin-resistant minimum inhibitory concentration ([MIC] ≥8 mg/L) *Pseudomonas aeruginosa, Klebsiella pneumoniae*, and *Acinetobacter baumannii*. However, when used in combination, clinically relevant concentrations of polymyxin B and SERMs displayed synergistic killing against the polymyxin-resistant *P. aeruginosa, K. pneumoniae*, and *A. baumannii* isolates as demonstrated by a ≥2–3 log10 decrease in bacterial count (CFU/ml) after 24 hours. The combination of polymyxin B with toremifene demonstrated very potent antibacterial activity against *P. aeruginosa* biofilms in an artificial sputum media assay. Moreover, polymyxin B combined with toremifene synergistically induced cytosolic green fluorescence protein release, cytoplasmic membrane depolarization, permeabilizing activity in a nitrocefin assay, and an increase of cellular reactive oxygen species from *P. aeruginosa* cells. In addition, scanning and transmission electron micrographs showed that polymyxin B in combination with toremifene causes distinctive damage to the outer membrane of *P. aeruginosa* cells, compared with treatments with each compound *per se*. In conclusion, the combination of polymyxin B and SERMs illustrated a synergistic activity against XDR Gram-negative pathogens, including highly polymyxin-resistant *P. aeruginosa* isolates, and represents a novel combination therapy strategy for the treatment of infections because of problematic XDR Gram-negative pathogens.

Keywords: SERMs, repositioning, Gram-negative, polymyxin, multidrug resistant

Introduction

The world is facing an enormous and growing threat from the emergence of extremely drug-resistant (XDR) bacterial "superbugs" that are resistant to all available antibiotics. The healthcare cost of antibiotic resistance to society is staggering. In the United States, antibiotic resistance costs an estimated $20 billion per year in direct healthcare costs, $35 billion per year in other societal costs, and >8 million additional days of extended hospital stay.[1,2] If bacteria continue developing resistance to multiple antibiotics at the present rate, at the same time as the antibiotic pipeline continues to dry up, there could be catastrophic costs to healthcare and society globally.[3]

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New antibiotic therapeutic strategies are urgently needed to treat infections caused by bacterial "superbugs," in particular Gram-negative *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae*. A novel, but rational, approach is repurposing FDA-approved drugs for alternative indications such as antimicrobial agents.[4,5]

Polymyxin B and colistin (polymyxin E) are lipopeptide antibiotics indicated as a last-line treatment for XDR Gram-negative bacterial infections (Fig. 1).[6] Worryingly, reports of polymyxin-resistant isolates are becoming more and more common, including very recent *Enterobacteriaceae* isolates with plasmid-carrying *mcr-1*.[7–9] Therefore, novel strategies to maintain the efficacy of these important last-line antibiotics against problematic XDR pathogens are crucial. An innovative strategy that is gaining momentum is the synergistic use of antibiotics with FDA-approved nonantibiotics.[10] In this novel approach, an FDA-approved nonantibiotic drug is combined with a specific antibiotic that enables it to exert its "off-target" antibacterial properties against the bacterial cell.

Selective estrogen receptor modulators (SERMs) tamoxifen, raloxifene, and toremifene are currently used for the treatment of advanced breast cancer and osteoporosis in premenopausal and postmenopausal women (Fig. 1).[11–14] Notably, SERMs have been previously reported to display direct antifungal, antiviral, and antiparasitic activities, making them perfect candidates for nonantibiotic–antibiotic synergy testing.[15–19] The purpose of this study was to investigate the *in vitro* synergistic antibacterial activity and mechanistic studies of SERMs in combination with polymyxin B against problematic XDR Gram-negative pathogens, including those resistant to polymyxins.

Methods

Materials

All compounds including polymyxin B (Catalogue No. 81334; ≥6,500 IU/mg) were purchased from Sigma-Aldrich (Melbourne, Australia). Polymyxin nonapeptide was produced through enzymatic cleavage of the fatty acyl diaminobutyric acid (Dab)[1] from polymyxin E and HPLC purified as previously described.[20,21]

Bacterial isolates

All bacterial strains used in this study are described in Table 1, including 13 mucoid and nonmucoid clinical isolates of *P. aeruginosa* selected from patients with acute exacerbations of cystic fibrosis.[22] A reference strain, *P. aeruginosa* ATCC 27853 (polymyxin B minimum inhibitory concentration [MIC] = 1 mg/L) (American Type Culture Collection, Rockville, MD), was examined. Resistance to polymyxin B was defined as MICs of ≥8 mg/L.[23] Four different *K. pneumoniae* isolates including polymyxin-susceptible and polymyxin-resistant isolates were also studied, including a reference strain *K. pneumoniae* ATCC 13883. A total of eight *A. baumannii* strains were examined in this study, including four polymyxin-susceptible and four polymyxin-resistant isolates.

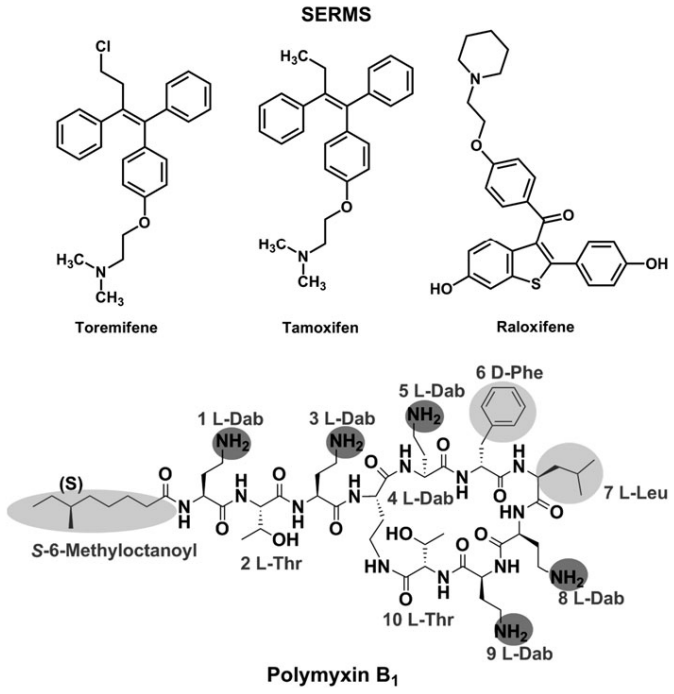

FIG. 1. Structures of the SERMs toremifene, tamoxifen, and raloxifene, and the lipopeptide antibiotic polymyxin B_1. The cationic side chains of the five diaminobutyric acid (Dab) residues of polymyxin B are highlighted by *darker gray circles* and the hydrophobic segments are highlighted by *lighter gray*. SERMs, selective estrogen receptor modulators.

Table 1. The Antimicrobial Activity of the SERMs (Tamoxifen TXN, Raloxifene RXN, and Toremifene TRN) and Polymyxin B (PMB) Alone, and the SERMs in Combination with Polymyxin B

	Isolates	MICs (mg/L)				FIC_{INDEX}[c]		
		PMB	TXN	RXN	TRN	PMB-TXN	PMB-RXN	PMB-TRN
Pseudomonas aeruginosa	Polymyxin-susceptible isolates							
	ATCC27853	1	>128	>128	>128	2	2	0.51
	FADDI-PA031 m[a]	1	64	64	>128	0.27	0.52	0.28
	FADDI-PA018 n/m[a]	1	>128	>128	>128	0.50	0.52	0.27
	FADDI-PA009 (L)[b] n/m	1	>128	>128	>128	0.38	0.50	0.25
	FADDI-PA008 (L) n/m	1	>128	>128	>128	0.50	0.38	0.50
	FADDI-PA007 n/m	2	>128	>128	>128	0.50	0.38	0.16
	FADDI-PA011 n/m	2	>128	>128	>128	0.31	0.31	0.16
	FADDI-PA020 m	2	>128	>128	>128	0.50	0.19	0.13
	Polymyxin-resistant isolates							
	FADDI-PA006 m	8	>128	128	>128	0.50	0.09	0.08
	FADDI-PA066 n/m	32	>128	64	>128	0.14	0.63	0.63
	FADDI-PA067 (S)[b] n/m	32	>128	>128	>128	0.07	0.38	0.05
	FADDI-PA070 n/m	64	>128	64	>128	0.06	0.38	0.09
	FADDI-PA065 m	>128	>128	>128	>128	0.14	0.14	0.04
Acinetobacter baumannii	Polymyxin-susceptible isolates							
	ATCC19606	1	>128	32	64	0.19	0.50	0.38
	FADDI-AB144	2	>128	128	16	0.13	0.38	0.56
	FADDI-AB150	2	>128	16	16	0.08	0.51	0.50
	FADDI-AB151	2	>128	128	128	0.08	0.16	0.25
	Polymyxin-resistant isolates							
	FADDI-AB148	8	>128	>128	>128	0.05	0.09	0.13
	FADDI-AB156	8	>128	64	64	0.06	0.50	0.08
	FADDI-AB143	16	>128	128	>128	0.05	0.13	0.13
	FADDI-AB060	128	>128	>128	>128	0.13	0.13	0.16
Klebsiella pneumoniae	Polymyxin-susceptible isolates							
	ATCC13883	0.25	>128	>128	>128	0.25	0.28	0.50
	FADDI-KP001	1	>128	>128	8	0.09	0.25	0.52
	Polymyxin-resistant isolates							
	FADDI-KP012	16	>128	>128	>128	0.28	0.50	2
	FADDI-KP003	>128	>128	>128	>128	2	2	2

[a]n/m, nonmucoid; m, mucoid.
[b]L, large colony; S, small colony.
[c]FIC = (MIC of polymyxin B in combination/MIC polymyxin B alone) + (MIC antineoplastic drug in combination/MIC antineoplastic alone); synergism FIC <0.5 [*green*]; addition FIC = 0.5–1.0 [*yellow*]; indifference FIC = 1–4 [*red*]; antagonism FIC ≥4 [not observed].
Color images available online at www.liebertpub.com/mdr

In vitro *antibacterial activity*

MICs were performed according to the Clinical and Laboratory Standards Institute guidelines.[24] MICs were determined for all isolates in three replicates on separate days using broth microdilution method in cation-adjusted Mueller–Hinton broth (CAMHB). Fractional inhibitory concentrations (FICs) were determined using the microdilution checkerboard method (FIC_{INDEX} values were interpreted as follows: synergism ≤0.5; additive = 1, indifferent >1 but <2 and antagonism ≥2).[25] If no endpoint MIC value could be determined because of the combination of resistance and the insolubility of the SERM compounds, the next highest MIC value was chosen for the FIC calculation (*i.e.*, if the MIC >128, an MIC = 256 was selected for the calculation). Therefore, the reported FIC values are essentially conservative estimates; in effect if it was possible to determine the endpoint MIC, then the FIC values would be lower (*i.e.*, better synergy). The static time–kill kinetics of polymyxin B *per se* and in combination with SERMs were investigated against select Gram-negative isolates, selected based on the determined FIC values.[26] Aliquots (~200 μl) of early log-phase bacterial suspensions were inoculated into 50 ml polypropylene tubes (Greiner Bio-one, Frickenhausen, Germany) containing 20 ml of CAMHB containing DMSO (2.5% v/v) and drug at the specified concentrations. Colonies were counted by a ProtoCOL-automated colony counter (Synbiosis, Cambridge, United Kingdom) after incubation for 24 hours at 35°C. The lower limit of counting was 20 CFU/ml.

Electron microscopy

Scanning and transmission electron microscopies (SEM and TEM) were conducted as previously described in detail after treatment against *P. aeruginosa* FADDI-PA067 cells with polymyxin B alone and in combination with toremifene for 2 hours.[27] Confocal imaging of the released cytosolic green fluorescence protein (GFP) was performed using *P. aeruginosa* strain Pa AH298-GFP expressing GFP under the control of the growth rate-dependent *rrnBp1* promoter that was kindly provided by Professor Philip S. Stewart.[28]

Nitrocefin assays

The outer membrane (OM) permeabilizing activity of the drugs was assessed using the β-lactamase nitrocefin assay.[29] In brief, the β-lactamase positive *P. aeruginosa* strain FADDI-PA067 (*P. aeruginosa* strains were tested for β-lactamase production using nitrocefin disk; Sigma-Aldrich) was subcultured on a nutrient agar at 35°C overnight. The culture (derived from a single colony) was grown to exponential phase and harvested by centrifugation (10,000 g; 10 minutes), washed twice in PBS, pH 7.2, and resuspended at an $OD_{600nm} = 0.50$. Nitrocefin (2 mM) was prepared in DMSO. The assay was performed in 96-well plates. The 200 μl reaction mixture consisted of 100 μl bacterial suspension, 96 μl drug (8 mg/L polymyxin B alone or in combination with 8 mg/L toremifene), and 4 μl of 2 mM nitrocefin solution. The absorbance (OD_{495nm}) was measured at room temperature for 140 seconds.

Artificial sputum media biofilm assay

The artificial sputum media (ASM) assays were performed with *P. aeruginosa* strain FADDI-PA067 in a 24-well plate format. ASM culture was freshly prepared as described by Kirchner *et al.* and filter sterilized.[29,30] *P. aeruginosa* strain FADDI-PA067 was subcultured on a nutrient agar and incubated at 35°C overnight. The overnight culture was diluted in CAMHB to an OD_{600nm} 0.05 ± 0.01 and then further diluted 1:100 in fresh ASM. A total volume of 1.8 ml was added to each well and incubated for 3 days under aerobic condition at 37°C before the addition of polymyxin B and toremifene alone or in combination for 24 hours. Viability was determined, wherein 100 μl of 0.02% (v/v) resazurin was added to each well and incubated for 2 hours at 37°C. Fluorescence of each well was measured using an ENVISION plate reader (PerkinElmer, Australia) at excitation 530 nm and emission 590 nm. Fluorescence was corrected by the subtraction of background noise. Cell viability was calculated as (mean fluorescence of treated biofilms/mean fluorescence of untreated control) × 100%. For each antibiotic concentration, four technical replicates were conducted.

Detection of cellular reactive oxygen species production

The production of reactive oxygen species (ROS) in *P. aeruginosa* strain FADDI-PA067 after antibiotic treatment was detected using the CellROX Green dye (Lifetechnologies, Melbourne, Australia). Fluorescence was measured using a Cary Eclipse Fluorescence spectrophotometer (Varian, Mulgrave, Australia) at excitation 485 nm, emission spectrum 490–600 nm. Slit widths were set to 5 nm for both the excitation and emission monochromators.

Cytoplasmic membrane depolarization assay

Cytoplasmic membrane depolarization was determined using cyanine dye diSC3-5 (3,3-dipropylthiadicarbocyanine iodide).[31] The assay was performed as previously described with slight modification.[32] Mid-log phase culture (1 ml) was harvested by centrifugation at 4,000 *g* for 10 minutes at room temperature and resuspended in 1 ml of assay buffer (5 mM HEPES, 20 mM glucose, pH 7.4). The bacteria suspension was diluted 100-fold using the assay buffer to reach the OD_{600nm} of 0.005. Cells were treated with 0.2 mM EDTA (pH 8.0) then with 0.4 μM diSC3-5 and incubated for 1 hour at room temperature. The uptake of diSC3-5 into bacterial cells was monitored using a Tecan Infinite® m1000 Pro Multi-mode reader. Then, 0.1 M KCl was added to equilibrate the cytoplasmic and external K^+ concentrations and 90 μl of the cell suspension was transferred to a 96-well black-walled plate (Corning #3609). After the fluorescence level stabilized, 10 μl of a concentration series of compounds was added into each well and the fluorescence intensity (excitation/emission: 620/670) was measured every minute for 40 minutes. Vehicle solution (10 μl H_2O) was used as a negative control in the assay.

Results

In vitro *antimicrobial synergy of polymyxin B in combination with SERMs*

Polymyxin B and the SERMs tamoxifen, raloxifene, and toremifene were screened for direct antimicrobial activity against a panel of polymyxin-susceptible and polymyxin-resistant clinical isolates of *P. aeruginosa*, *K. pneumoniae*, and *A. baumannii* (Table 1). The polymyxin B–SERM combinations displayed excellent synergy against the polymyxin-resistant isolates of each species (FIC_I 0.04–0.38). Except for

the appreciable activity of toremifene against *K. pneumoniae* FADDI-KP001 (MIC 8 mg/L), the SERMs *per se* were not active against any of the *P. aeruginosa*, *K. pneumoniae*, and *A. baumannii* isolates (MICs 64 to >128 mg/L). The combination of polymyxin B with tamoxifen displayed synergistic activity against 8 of the 13 *P. aeruginosa* isolates, including all 5 polymyxin-resistant isolates, the 8 *A. baumannii* isolates, and 3 out of 4 *K. pneumoniae* isolates. The combination of polymyxin B with raloxifene displayed synergistic activity against eight *P. aeruginosa* isolates, five *A. baumannii* isolates, and two *K. pneumoniae* isolates. The combination of polymyxin B with toremifene displayed synergistic activity against 10 out of 13 *P. aeruginosa* isolates, 6 *A. baumannii* isolates, but none of the *K. pneumoniae* isolates. To probe whether the synergy between polymyxin B and the SERMs is a result of the permeabilizing activity of polymyxin on the Gram-negative OM that allows the SERMs to enter the cells and reach their intracellular targets, we tested polymyxin nonapeptide in combination with tamoxifen against two polymyxin-resistant *P. aeruginosa* strains (FADDI-PA067 and FADDI-PA070) and the polymyxin-sensitive strain *P. aeruginosa* ATCC 278853. Polymyxin nonapeptide has the *N*-terminal fatty acyl Dab[1] segment removed and consequently lacks the direct bactericidal activity of the mature polymyxin molecule, although it retains the OM permeabilizing activity and is often employed as a sensitizer for other antibiotics.[21] The polymyxin nonapeptide–tamoxifen combination was inactive against *P. aeruginosa* strains FADD-PA067 and FADDI-PA070, whereas good synergy (FIC = 0.18) was observed against *P. aeruginosa* ATCC 278853.

Antibacterial killing kinetics of the polymyxin B–SERM combinations

The synergistic killing activity of the polymyxin B–SERM combinations in static time–kill studies was assessed against seven Gram-negative clinical isolates (Fig. 2). Clinically relevant concentrations of the drugs were used in the time–kill experiments.[6,12–14] The antipseudomonal activity of the polymyxin B–SERM combinations was assessed against FADDI-PA070 (polymyxin B MIC 64 mg/L; tamoxifen >128 mg/L), FADDI-PA065 (polymyxin B MIC >128 mg/L; tamoxifen >128 mg/L), FADDI-PA067 (polymyxin B MIC 32 mg/L; toremifene >128 mg/L), and FADDI-PA006 (polymyxin B MIC 8 mg/L; raloxifene >128 mg/L). Monotherapy with polymyxin

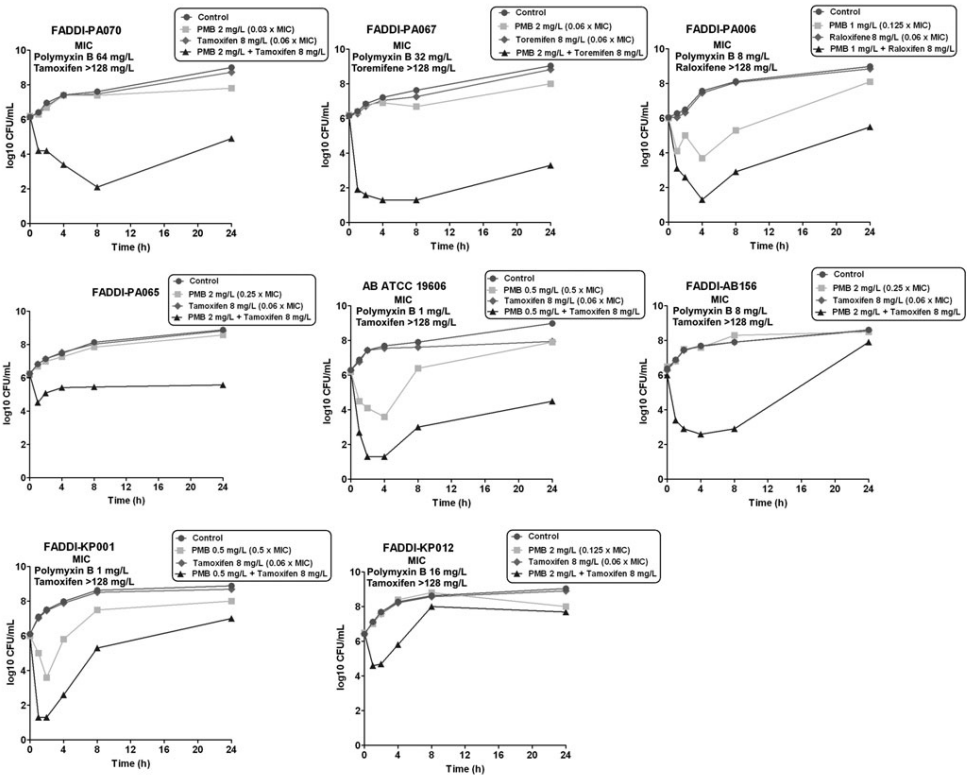

FIG. 2. Time–kill curves for polymyxin B–SERM combinations against polymyxin-susceptible and polymyxin-resistant clinical isolates *of Pseudomonas aeruginosa, Klebsiella pneumoniae*, and *Acinetobacter baumannii*.

B or each SERM exhibited no antibacterial activity against the *P. aeruginosa* isolates up to 24 hours with the bacterial killing curves indistinguishable from that of the control.

The polymyxin B–tamoxifen combination displayed a ~4log10 CFU/ml decrease against *P. aeruginosa* FADDI-PA070 over 24 hours as compared with the control. The polymyxin B–tamoxifen combination displayed a ~3.2 log10 CFU/ml decrease against *P. aeruginosa* FADDI-PA065 over 24 hours as compared with the control. The polymyxin B–toremifene combination displayed a decrease in *P. aeruginosa* FADDI-PA067 bacterial count of ~5.7log10 CFU/ml over 24 hours as compared with the control. The polymyxin B–raloxifene combination displayed a decrease in *P. aeruginosa* FADDI-PA006 bacterial count of ~3.5log10 CFU/ml over 24 hours as compared with the control.

The killing kinetics of polymyxin B–tamoxifen combinations against *A. baumannii* were assessed against a polymyxin-susceptible strain ATCC 19606 (polymyxin B MIC 1 mg/L; tamoxifen MIC >128 mg/L) and a polymyxin-resistant XDR clinical isolate FADDI-AB156 (ST22 containing OXA-23, OXA-66, TEM-116, PER-1, and ADC-29)[33] (polymyxin B MIC 8 mg/L; tamoxifen >128 MIC mg/L). The polymyxin B–tamoxifen combination performed well against *A. baumannii* ATCC 19606 and caused a decrease in bacterial count of ~4.5log10 CFU/ml over 24 hours as compared with the control. However, despite the good FIC_I (0.07), the polymyxin B–tamoxifen combination displayed moderate activity against the XDR polymyxin-resistant *A. baumannii* FADDI-AB156 isolate, with a decrease of ~5log10 CFU/ml over 2–8 hours; although extensive regrowth occurred at 24 hours.

Finally, we examined the killing kinetics of the polymyxin B–tamoxifen combination against the polymyxin-susceptible *K. pneumoniae* FADDI-KP001 isolate (polymyxin B MIC 1 mg/L; tamoxifen >128 MIC mg/L) and the polymyxin-resistant *K. pneumoniae* FADDI-KP012 isolate (polymyxin B MIC 16 mg/L; tamoxifen >128 MIC mg/L). Against *K. pneumoniae* FADDI-KP001, the polymyxin B–tamoxifen combination decreased the bacterial count ≥6 log10 CFU/ml over 1–2 hours, followed by extensive regrowth from 4 to 24 hours. Irrespective of the good FIC_I (0.26), the polymyxin B–tamoxifen combination displayed poor activity against *K. pneumoniae* FADDI-KP012, seen as a decrease in bacterial count of ~3log10 CFU/ml over 1–2 hours, followed by extensive regrowth from 4 to 24 hours.

Overall, the polymyxin B–toremifene combination displayed the best killing kinetics and as such was selected for the following antipseudomonal mechanistic studies. To assess whether the rebound growth seen at 24 hours relates to polymyxin B resistance, we recovered the cells from an isolate of each species at 24 hours and tested the polymyxin B MIC. In the case of *K. pneumoniae* FADDI-KP001 (polymyxin B MIC 1 mg/L), the polymyxin MIC of the rebound cells did not change significantly, 2 mg/L; for *A. baumannii* FADDI-AB156 (polymyxin B MIC 8 mg/L), the polymyxin MIC of the rebound cells did not change, 8 mg/L; for *P. aeruginosa* FADDI-PA006 (polymyxin B MIC 8 mg/L), the polymyxin MIC of the rebound cells increased to 32 mg/L. These results suggest that the rebound growth seen at 24 hours with *P. aeruginosa* FADDI-PA006 is likely because of polymyxin B resistance, whereas with *K. pneumoniae* FADDI-KP001 and *A. baumannii* FADDI-AB156, the regrowth at 24 hours is likely because of resistance to the SERM.

Antipseudomonal activity of the polymyxin B–toremifene combination in sputum

The antipseudomonal activity of polymyxin B and toremifene *per se* and in combination against the *P. aeruginosa* isolate FADDI-PA067 (polymyxin B MIC 32 mg/L; toremifene >128 mg/L) was assessed in an ASM assay (Fig. 3). Although each drug alone was completely inactive, the polymyxin B–toremifene combination remarkably reduced cell viability to ~15% of the untreated control cells under sputum-like conditions.

Imaging the OM permeabilizing activity of polymyxin B–toremifene combination against P. aeruginosa

SEM and TEM imaging of *P. aeruginosa* FADDI-PA067 cells treated with polymyxin B and toremifene alone and in combination for 2 hours demonstrated extensive ultrastructural changes (Fig. 4). Our TEM images revealed that treatment with each compound alone or in combination produces ultrastructural damage to *P. aeruginosa* cells, including severe damage of the cell envelope, leakage of cytoplasmic material, and coagulation of the cytoplasmic matrix. In the SEM images, the treated cells appeared swollen and corrugated with membrane blebbing. These structural alterations clearly indicate a loss of cellular integrity. Similarly, confocal imaging of the *P. aeruginosa* strain AH298-GFP proved that the combination was more effective at producing damage to the cell envelope than each drug alone, as measured by the release of cytosolic GFP (Fig. 5).

The polymyxin B–toremifene combination induces increased nitrocefin uptake across the OM and production of ROS in P. aeruginosa

To further confirm that the combination of polymyxin B–toremifene induced cell envelope damage, the nitrocefin assay

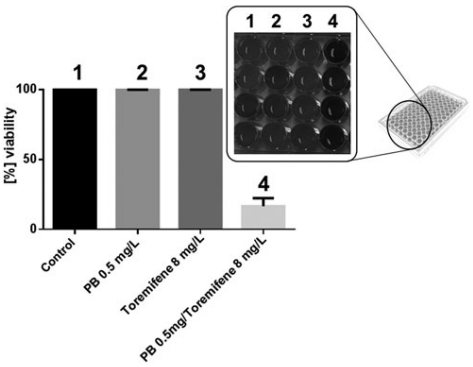

FIG. 3. Antimicrobial activity of the polymyxin B–toremifene combination against *P. aeruginosa* FADDI-PA067 in artificial sputum media. Data points are plotted as the mean ± SD of four independent measurements. The *top right*-hand corner inset graphics in each bar graph show the raw data from the resazurin fluorescence plate assay; numbers 1–4 correspond to the numbering for each condition in the adjacent bar graph. The replicates for each condition are organized vertically in the 96-well plate.

was employed using the β-lactamase positive strain *P. aeruginosa* FADDI-PA067 (Fig. 6A). The assay measures periplasmic β-lactamase activity using nitrocefin, a chromogenic cephalosporin substrate analogue, which ordinarily permeates the OM slowly.[34] In the presence of OM permeabilizing agents, nitrocefin can more readily penetrate the OM and is hydrolyzed to its colormetric form by intracellular β-lactamase. The combination of polymyxin B–toremifene was significantly more effective at permeabilizing the OM than either of the drugs *per se*, seen from the increased β-lactamase-mediated conversion of nitrocefin (Fig. 6A).

ROS production is believed to be a common mechanism, whereby bacteriacidal antibiotics induce bacterial killing.[35–37]

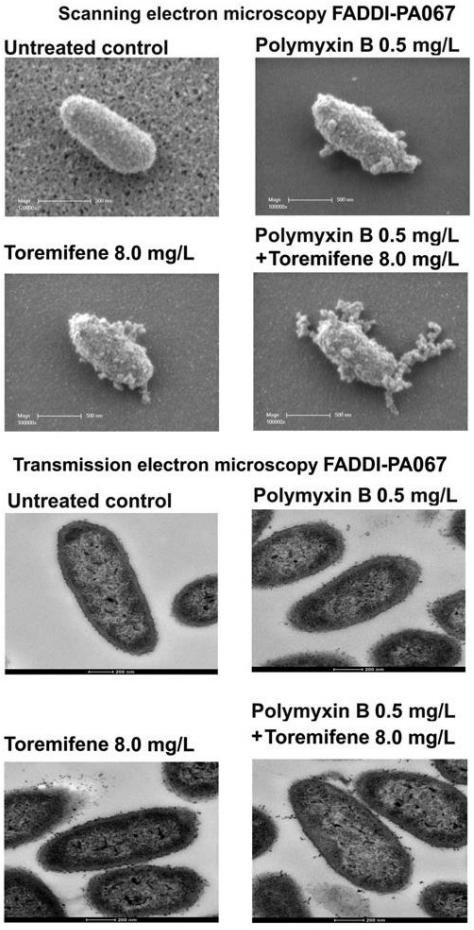

FIG. 4. Electron microscopy images of the *P. aeruginosa* isolate FADDI-PA067 (polymyxin B MIC 32 mg/L; toremifene >128 mg/L) treated with the polymyxin B–toremifene combination.

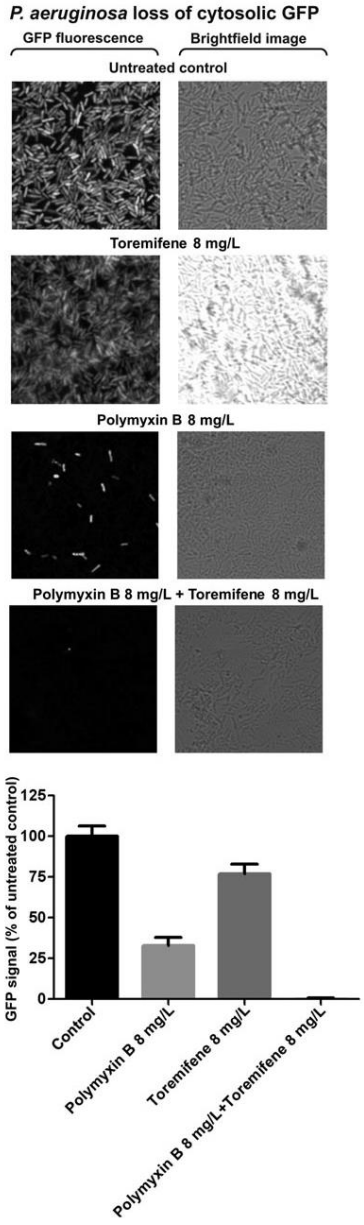

FIG. 5. *Top panels:* confocal microscopy images of cytosolic GFP release from *P. aeruginosa* AH298-GFP treated with the polymyxin B–toremifene combination. *Bottom panel:* Bar graph showing the GFP signal as the percentage of the untreated control ($n = 4$). GFP, green fluorescence protein.

Figure 6B shows that the combination induced a greater increase of ROS production in *P. aeruginosa* FADDI-PA067 cells than the treatment with each drug alone, as seen by the increase in fluorescence emission of the oxidative stress-sensitive dye CellROX Green.

The polymyxin B–toremifene combination induced cytoplasmic membrane depolarization in P. aeruginosa

The combination of toremifene and polymyxin B (16 μg/ml of each compound) showed a stronger membrane depolarizing effect on *P. aeruginosa* FADDI-PA067 cells than treatment with each compound alone (at 16 μg/ml) (Fig. 6C). Notably, toremifene displayed a significantly greater membrane depolarizing effect than polymyxin B. Triton 10× at 0.5% was used as a positive control and carbapenem, meropenem, as a negative control of membrane depolarization. Meropenem shows no significant fluorescent changes as expected with the mode of action targeting the inhibition of cell wall synthesis, rather than being membrane active.

Discussion

The dawn of the "postantibiotic era" is approaching because of the rise of antibiotic-resistant "superbugs" and the fact that the pharmaceutical industry has not developed new antibiotic classes for more than a decade. Resistance to the last-line polymyxins is of particular concern, as bacteria can now rapidly acquire polymyxin resistance through the wide-spread dissemination of the *mcr-1* plasmid.[38 40] Repurposing of compounds from the vast FDA-approved drug reservoir is a highly viable, low-risk strategy for the development of novel antimicrobial therapies, particularly compared with *de novo* drug discovery.[4]

This study demonstrates that synergistic antibacterial activity against problematic XDR Gram-negative "superbugs" can be found when combining antineoplastic SERM drugs with polymyxin B. Notably, the polymyxin B–SERM combinations achieved 65% synergy coverage across the 75 isolates tested, which is most encouraging. The less than complete coverage is likely because of variability in susceptibility to the antibacterial mechanisms involved across Gram-negative isolates. The SERM–polymyxin combinations produced excellent antibacterial killing kinetics against polymyxin-resistant *P. aeruginosa*, *A. baumannii*, and *K.*

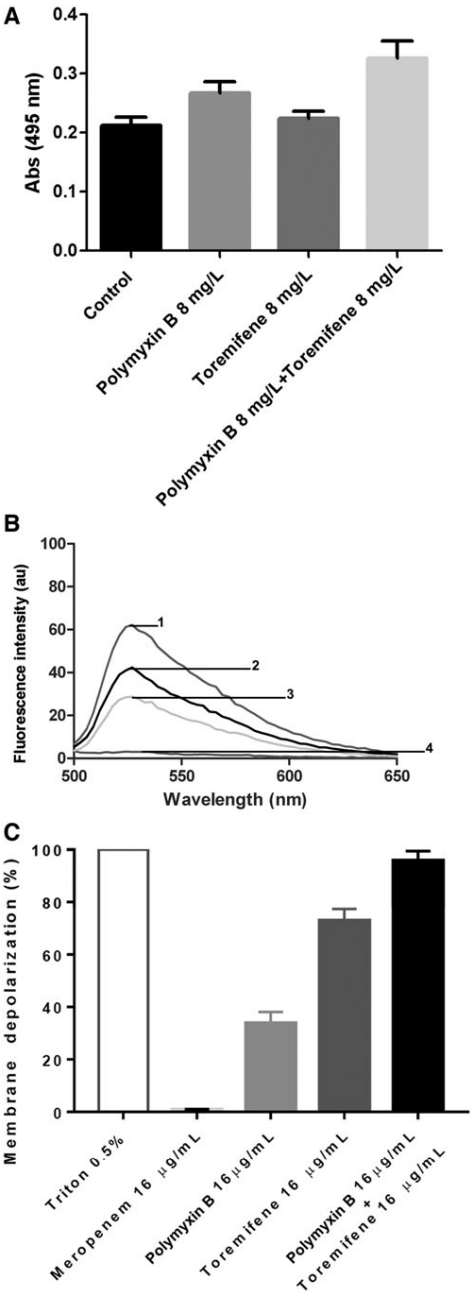

FIG. 6. **(A)** OM permeabilizing activity of polymyxin B and toremifene alone and in combination against *P. aeruginosa* FADDI-PA067. The β-lactamase activity was measured by the nitrocefin assay for 140 seconds. The results presented are the mean of four independent measurements ± standard deviation. **(B)** ROS production in *P. aeruginosa* FADDI-PA067 cells after treatment measured by the increased fluorescence emission of the oxidative stress-sensitive dye CellROX Green. Treatments: (1) untreated cells, (2) polymyxin B 2 mg/L, (3) toremifene 8 mg/L, (4) combination of polymyxin B 2 mg/L+toremifene NP 8 mg/L. **(C)** Membrane depolarization of *P. aeruginosa* FADDI-PA067 cells calculated as a measurement of fluorescence intensity of diSC3-5 dye. Polymyxin B+toremifene 32 μg/ml = 16 μg/ml of each compound. All treatment groups were normalized to Triton-X (0.5%) producing 100% membrane depolarization. All conditions, $n = 6$, error bars ± SD. OM, outer membrane; ROS, reactive oxygen species.

pneumoniae and very potent antipseudomonal activity under sputum-like conditions (Figs. 2 and 3).

SERMs are nonsteroidal estrogen receptor antagonists mostly used for chemotherapy and chemoprevention of breast cancers.[11–13] Besides their antineoplastic activity, SERMs have been found to possess direct antimicrobial activities.[15–18] Tamoxifen and toremifene have been shown to possess potent activity against *Cryptococcus neoformans* and *Candida albicans* by interfering with calcineurin signaling through binding to calmodulin and calmodulin-like proteins.[15,17] With respect to the antifungal mode of action of SERMs, multiple mechanisms have been inferred, including membrane damage, inhibition of membrane peroxidation, cell cycle arrest, and, as already mentioned, interference with calcium homeostasis.[15,17] Moreover, toremifene has been reported to display direct inhibitory activity against Ebola virus and inhibits biofilm formation by *Candida* spp., *P. aeruginosa*, *Staphylococcus aureus*, and *Staphylococcus epidermidis*.[18,41–43] Raloxifene has been shown to diminish *P. aeruginosa* virulence in a *Caenorhabditis elegans* infection model.[41] Furthermore, raloxifene treatment produces a dose-dependent reduction in pyocyanin production by *P. aeruginosa* PAO1 and PA14.[41]

The Gram-negative OM is a formidable barrier, especially against hydrophobic drugs such as the SERMs.[44] The antimicrobial action of polymyxins is mediated through a direct interaction with the lipid A component of the lipopolysaccharide (LPS), which leads to a disruption of the OM barrier.[6] The cationic L-α,γ-Dab residues of the polymyxin molecule produce an electrostatic attraction to the negatively charged lipid A phosphate groups, displacing the divalent cations (Mg^{2+} and Ca^{2+}).[6] The displacement leads to the disorganization of the LPS leaflet, enabling the insertion of the hydrophobic tail and the hydrophobic side chains of amino acids 6 and 7 of the polymyxin molecule into the OM.[45] Hence, one obvious mechanism of the synergy between SERMs and polymyxin B may involve the ability of polymyxin B to permeabilize the OM, thereby allowing the SERMs to enter the cell and exert their antibacterial action on intracellular targets. Evidence in support of this model stems from our experiments with polymyxin nonapeptide, which showed excellent synergy in combination with tamoxifen against the polymyxin-susceptible strain *P. aeruginosa* ATCC 278853.

Notably, the nonapeptide–tamoxifen combination was inactive against the two polymxyin-resistant *P. aeruginosa* strains tested; this is likely because of the requirement for added hydrophobicity within the polymyxin scaffold to permeabilize the OM of resistant isolates.[46] However, it is unlikely that a single mode of action is responsible for the antibacterial synergy, particularly in view of the aforementioned multitude of cellular effects that SERMs mediate. An alternative mechanism, which we purport, is that the antibacterial synergy of the combination is also a result of their combined OM activity. As already mentioned, the primary mode of action of polymyxins involves disruption of the Gram-negative OM, similarly the SERMs are considered to be membrane-active drugs.[47–50] Tamoxifen is known to interact with lipids in biomembranes and cause ultrastructural changes, which may result in cell lysis.[43,48] Notably, triaryl butane analogues of tamoxifen have been shown to induce sodium and potassium efflux from Gram-negative cells and loss of transmembrane potential.[47]

Similarly, our findings demonstrated that the polymyxin B–toremifene combination and toremifene treatment *per se* produce an appreciable depolarization of the cytoplasmic membrane of *P. aeruginosa* cells (Fig. 6C). Additional effects of tamoxifen that have been suggested to reflect its direct action on biomembranes include its ability to change the morphology of the breast tumor cell membranes[48]; other biomembrane-related effects include hemolysis,[49] mitochondrial swelling,[51] and proton leakage from the mitochondrial inner membrane, resulting in depolarization of membrane potential.[52,53] Accordingly, the accentuated ultrastructural alterations we observed in *P. aeruginosa* cells treated with the polymyxin B–toremifene combination compared with treatment with either of the drugs alone are potentially a consequence of their combined disruptive membrane activity (Fig. 4). Similar to our SEM and TEM findings, electron microscopy studies of the membrane effects of tamoxifen on a strain of *Bacillus stearothermophilus* revealed changes in the membrane structure from asymmetric to symmetric and the appearance of fractures in the cell wall.[50] The synergistic damaging effect of the polymyxin B–toremifene combination was corroborated by the GFP release and nitrocefin assay data (Figs. 5 and 6A). Notably, both polymyxin B and toremifene alone and in combination produced a marked increase in cellular ROS in *P. aeruginosa* cells, which suggests that the latter could also contribute to the potent killing effect (Fig. 6B).

In summary, our study demonstrates that the combination of polymyxin B with SERMs might offer a new opportunity to treat otherwise untreatable XDR Gram-negative infections. *P. aeruginosa* in particular is intrinsically resistant to antibiotics, and is noted as requiring an urgent need for new antipseudomonal therapeutic options.[54] From a practical standpoint, this *in vitro* study suggests that the "off-the-shelf" SERMs represent a potentially novel antibiotic class for further antibiotic drug discovery and development against multi-drug resistant (MDR) Gram-negative "superbugs." The SERM concentrations used in this study are achievable in patients through oral administration and are well tolerated.[55–58] The high oral bioavailability of tamoxifen and toremifene together with the availability of pre-existing libraries of analogues from structure–activity relationship studies performed during their development as breast cancer therapeutics could expedite the identification of novel SERMs with superior antibacterial activities.

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Disclosure Statement

No competing financial interests exist.

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