Intracellular genetic network involving anti-microbial resistance expression in *Klebsiella pneumoniae* strains which acquire carbapenem resistance *in-vivo* and *in-vitro*

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Intracellular genetic network involving anti-microbial resistance expression in *Klebsiella pneumoniae* strains which acquire carbapenem resistance *in-vivo* and *in-vitro*

Directed by Professor Dongeun Yong

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ABSTRACT

Intracellular genetic network involving anti-microbial resistance expression in *Klebsiella pneumoniae* strains which acquire carbapenem resistance *in-vivo* and *in-vitro*

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(Directed by Professor Dongeun Yong)

Antibiotic resistance is an important health crisis worldwide. It is predicted that by the year 2050, the number of deaths due to antimicrobial resistant bacteria is going to reach an all-time high of 10 million a year. The magnitude of impact of multi-drug resistant bacteria on lives of people has forced us to find novel drug targets and mechanisms to control these pathogens at the earliest. The pace of resistance acquisition by the bacteria has surpassed that of finding newer antibiotics. *Klebsiella pneumoniae*, an opportunistic pathogen, is well-known for its nosocomial pathogenicity by displaying resistance to most antibiotics commercially available, thereby, limiting treatment options. The aim of my dissertation was to find novel resistance mechanisms in clonally related carbapenemase non-producing carbapenem-resistant *K. pneumoniae* strains obtained from a patient after meropenem treatment. In addition, finding a reliable method for porin detection in carbapenem-resistant *K. pneumoniae* strains.

Chapter I provides a brief overview on the history of antibiotics, antibiotic drug targets and resistance mechanisms, along with β-lactams and mechanisms of carbapenem resistance in *K. pneumoniae*.

Chapter II describes finding the cause of resistance in a carbapenemase non-producing carbapenem-resistant *K. pneumoniae* clinical isolate that showed strong three dimensional bioassay test positive indicating the presence of carbapenemase enzyme. Radiation mediated mutagenesis was used to render the resistant strain susceptible. The cause of positive 3D bioassay was attributed to the presence of $bla_{\text{CMY-10}}$ gene in the plasmid of the isolate which is an AmpC β-lactamase gene. Complementation of $bla_{\text{CMY-10}}$ gene into clinical isolates and outer membrane protein (OMP) mutants concluded that the carbapenem resistance occurrence in $bla_{\text{CMY-10}}$-carrying *K. pneumoniae* isolates was due to the loss of both OmpK35 and OmpK36 porins.

Chapter III illustrates novel mechanisms that bring about meropenem susceptibility in *K. pneumoniae* acquired carbapenem resistance *in-vivo* and *in-vitro*. The putative candidate genes were short-listed using whole genome analysis, transcriptome analysis and a functional gene network called KlebNet. Complementation of *KPHS_33600* (MFS transporter) and *KPHS_46730* (*garL*) genes showed decrease in meropenem MIC (from ≥32 μg/ml to 8 μg/ml) in *in-vivo* resistant strain K56, and *in-vitro* resistant strain K26M, respectively. The complemented strains did not show reduction in fitness when grown in LB broth and *Galleria mellonella* larvae successfully recovered when treated with meropenem when infected with the *KPHS_33600* and *garL* complemented strains. Possible mechanism of action has also been illustrated using transcriptome data obtained from the complemented strains.

Chapter IV compares the use of different methods for OMP detection i.e. MALDI-TOF MS, SDS-PAGE, WGS and transcriptome data analysis. At present, SDS-PAGE is the gold standard for OMP detection. We found discrepancy in OMP detection using SDS-PAGE in *K. pneumoniae*, therefore, we compared the results using the methods mentioned above. In addition, peptide sequencing was carried out to confirm the SDS-PAGE bands. OmpK35 could not be detected using SDS-PAGE and MALDI-TOF MS. However, the results obtained from both these methods were identical, concluding that MALDI-TOF MS can replace SDS-PAGE. RNA analysis could not accurately confirm due to lower level expression of mutated genes. Whole genome and/or PCR followed by Sanger sequencing could accurately detect the OMPs, thereby making them the most reliable methods for OMP detection of *K. pneumoniae* clinical isolates.

In conclusion, this dissertation is focused on finding resistance mechanisms, cause of resistances and reliable detection method for OMPs in carbapenem-resistant *K. pneumoniae* clinical isolates. Since *K. pneumoniae* is not very well studied as *Escherichia coli* or PAO-1 of *Pseudomonas aeruginosa*, there are several uncharacterized genes that are of research interest. Characterization of *KPHS_33600*, one of such uncharacterized transporter gene, can provide further insight into its functional relationship with meropenem susceptibility.

Key words: *Klebsiella pneumoniae*, *bla*$_{\text{CMY-10}}$, radiation-mediated mutagenesis, whole genome sequencing, KlebNet, transcriptome analysis, outer membrane protein

Chapter I

Introduction to antibiotics and *Klebsiella pneumoniae*

I. A BRIEF HISTORY OF ANTIBIOTICS

The word 'antibiotic' was first used by Selman Waksman in 1941 to describe any molecule produced by one microbe that antagonizes other microbes' growth.[1] They are antimicrobial drugs that are used to treat and prevent bacterial infections. The mode of action may vary, i.e., growth inhibition or bacterial killing. Traditionally, the term antibiotics was restricted to naturally available products from other micro-organisms, however, the term is now generalized to both natural and synthetically manufactured drugs as well.

The rise of modern 'antibiotic era' is usually associated with the names Paul Ehrlich and Alexander Fleming. While Ehrlich discovered Salvarsan, that cured syphilis, in 1909, Fleming's serendipitous discovery of penicillin occurred in 1928.[2] Prior to the use of penicillin in the 1940's, infections such as rheumatic fever or gonorrhea could not be treated effectively. Discovery of these antibiotics was the greatest advancement in the field of therapeutic medicine. They increased the life expectancy by successfully treating infections[3] and by treating the war wounded soldiers who were prone to higher risks of infections. In the years to follow, several new antibiotics were discovered. The years between 1950's and 1970's saw the discovery of novel classes of antibiotics, with a rapid decline in discovery rate since then[2]. Only two new classes have been discovered in the late 1990's, namely, oxazolidinone and cyclic lipopeptide.[4]

II. ANTIBIOTIC TARGETS AND RESISTANCE MECHANISMS

Antibiotics are categorized into five groups according to their microbial targets. These groups are inhibitors of cell wall synthesis, inhibitors of protein synthesis, inhibitors of membrane function, anti-metabolite activity (folate pathway inhibitors) and inhibitors of nucleic acid synthesis. Antibiotics are non-toxic to humans because these targets are either non-existent or different in eukaryotes. Figure 1.1 provides a representation of various antibiotic targets and resistance mechanisms.

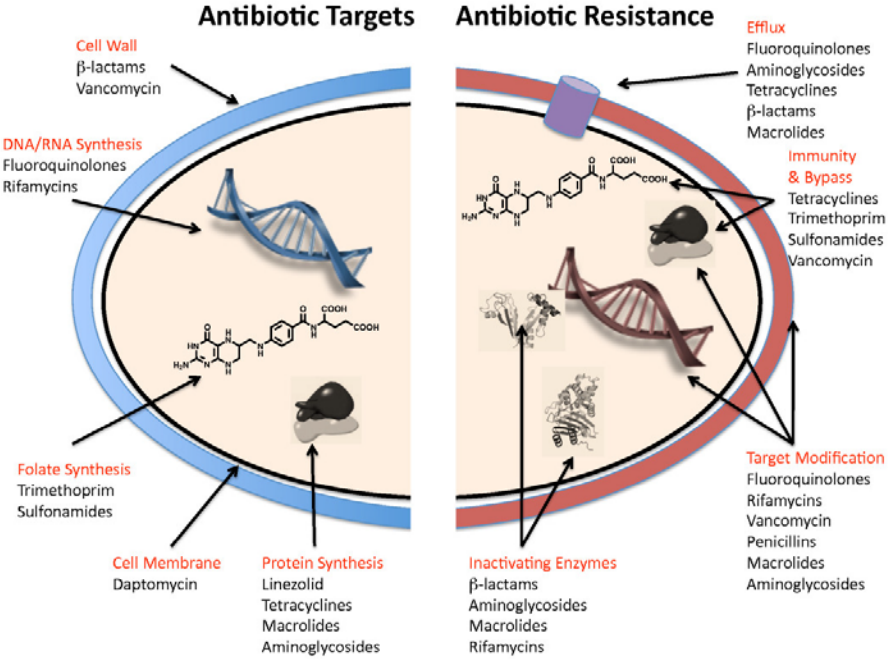

Figure 1.1. Antibiotic targets and resistance mechanisms (replicated from Wright, G.D. *BMC* Biology 2010 8:123)[5].

Antibiotic resistance can occur through one of the following mechanisms:

1. Drug extrusion by over-expressed efflux pumps: They are a large family of protein pumps that extrude drugs and have broad substrate specificities. The five major families of efflux pumps are resistance nodulation division (RND) family, major facilitator superfamily (MFS), adenosine triphosphate-binding cassette (ABC) superfamily, multidrug and toxic compound extrusion (MATE) family and small multidrug resistance (SMR) family.
2. Drug target modification: This usually occurs because of mutation of the drug target site, thereby rendering the antibiotic unavailable for bacterial killing.
3. Enzyme inactivation: Enzymes that inactivate the antibiotics are produced by the bacteria. Ex. β-lactamases, cephalosporinases, extended-spectrum β-lactamases (ESBLs) etc.
4. Immunity: In this case, proteins are bound to antibiotics or their targets making them unavailable for target binding.

III. β-LACTAM ANTIBIOTICS

β-lactams are broad spectrum antibiotics that contain beta-lactam ring in their structure. They include penicillins, cephalosporins, monobactams and carbapenems. β-lactams inhibit cell wall synthesis by binding to the penicillin binding protein (PBP) in the cytoplasmic membrane of the bacteria.

Carbapenems are broad spectrum beta-lactam antibiotics increasingly used these days to treat ESBL producing bacterial isolates. Thienamycin was the first naturally occurring carbapenem that was derived from *Streptomyces cattleya*.[6] The structure of the beta-lactams makes them resistant to ESBLs and cephalosporinase enzymes.

IV. IMPACT OF CARBAPENEM RESISTANCE

Antibiotic resistance and its associated infectious diseases are one of the important causes of global health crisis.[7] In addition to increased resistance to existing agents, development of new antibiotics are lagging behind.[2] According to CDC in United States, more than 2 million people are infected by antibiotic resistant bacteria annually, with 23,000 deaths. Billions of dollars are being spent on medical needs due to these multi-drug resistant bacteria (http://www.cdc.gov/drugresistance/).

Among antibiotics, carbapenems play a critical role since they are the last line of defense during treatment. Carbapenems are broad spectrum β-lactam antibiotics and stable when exposed to cephalosporinases and ESBLs. They are used to treat most of gram-negative and gram-positive bacteria except methicillin-resistant *Staphylococcus aureus*. Carbapenems easily enter gram-negative bacteria through outer membrane proteins (OMPs) called porins. In the periplasmic space, they permanently acylate the penicillin-binding proteins (PBPs), which is important for bacterial cell wall synthesis, thus leading to autolysis of the cell[5]. Most commonly

known carbapenems are imipenem, meropenem, ertapenem and doripenem. When bacteria become resistant to these carbapenems, it is worrisome because to-date there are no new antibiotics that can kill these bacteria.

V. MOLECULAR RESISTANCE MECHANISMS TO CARBAPENEM

1. Carbapenemase enzymes

Enterobacteriaceae family are the causative agents of most nosocomial as well as community acquired infections. In addition, they have also acquired resistance to prescribed antibiotics.[8] As a last resort, the clinicians increased the use of carbapenems to treat multi-drug resistant strains such as ESBL- or AmpC β-lactamase producers. As predicted, similar to other antibiotics, there was an increase in carbapenem-resistant *Enterobacteriaceae* (CRE)[9,10] which is a problem from over a decade now.

CRE carrying carbapenemase genes inactivate carbapenem and makes it unavailable for bacteria killing.[11] Carbapenemases are of two types - metallo β-lactamases such as IMP, VIM, NDM and non-metallo β-lactamases such as KPC, GES and OXA-48.[12] The emergence of carbapenem-resistance occurs in patients on long term carbapenem treatment.[13-15] It has been found that the strains susceptible to carbapenems acquire resistance after treatment. These studies indicate that the

bacteria are capable of mutating *in-vivo* to adapt to the antibiotic stress offered by carbapenems.

2. Carbapenemase-producing *K. pneumoniae*

To date, several nosocomial outbreaks have been reported due to spread of *K. pneumoniae* carrying plasmid-borne carbapenemase genes worldwide. A study has also showed cross-species transmission of carbapenemase-producing gene mediated through plasmid.[16] Therefore, *K. pneumoniae* isolates are most common and notorious in hospital-related infections. *K. pneumoniae* in itself is an opportunistic pathogen and a common nosocomial microbe. The mortality rate associated with it is as high as 50%.[17] Studies have shown numerous outbreaks of KPC-producing *K. pneumoniae* strains[18-23] and they are disseminated all over the world.[12] Some of the risk factors include administration of broad-spectrum antimicrobial agents, non-compliance with infection control practices, use of invasive medical procedures-catheterization, prolonged stay in ICU, incompletely developed immune system and low birth weight in preterm infants.[14] Moreover, these infections lead to increase in length of hospital stay as well as higher medical costs.

3. Carbapenemase non-producing *K. pneumoniae*

There are also carbapenemase non-producing carbapenem-resistant *K. pneumoniae* that do not carry carbapenemase gene and yet resistant to carbapenems.[24]

They are usually assigned as carbapenemase-producers by automated methods thus hindering detection, antibiotic therapy and infection control measures. The causes of resistance in these strains are derepression of inherent AmpC-encoding gene, acquisition of exogenous plasmid-borne cephalosporinase or ESBL genes, porin permeability reduction or expression of high level ESBL or AmpC cephalosporinase combined with porin alteration.[11] Few nosocomial epidemic outbreaks of carbapenemase non-producing carbapenem-resistant *K. pneumoniae* are registered which is a cause for concern.[25-28] It is a misconception that carbapenemase non-producers are not a threat as compared to carbapenemase-producers.

The aim of my dissertation was to find novel resistance mechanisms in clonally related carbapenemase non-producing carbapenem-resistant *K. pneumoniae* strains obtained from patient after meropenem treatment. As described earlier, porins are responsible for antibiotic entry into the bacteria. It is found to be one of the most common mechanisms by which bacteria gains resistance to anti-microbial agents.[8,12,13] So a part of my study is focused to find sensitive and reliable way to accurately detect the OMPs by comparing MALDI-TOF MS, SDS-PAGE, whole genome sequencing and transcriptome analysis.

CHAPTER II

Cause of carbapenem resistance in *bla*$_{\text{CMY-10}}$-carrying *K. pneumoniae* strains

Cause of carbapenem resistance in *bla*$_{CMY-10}$-carrying *Klebsiella pneumoniae* strains

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I. INTRODUCTION

Presently, there is a worldwide increase in carbapenem-resistant bacteria and extensive research to uncover more information on resistance acquisition and/or novel resistance mechanisms is being pursued. Carbapenem resistance exhibited by the bacteria maybe due to the presence of carbapenemase genes or ESBLs accompanied by porin loss or over-production of AmpC β-lactamase accompanied by porin loss.[29] Carbapenemase-producing gram-negative isolates are usually determined using one or more of the following methods such as PCR, disk diffusion susceptibility (DDS) testing, double disk synergy testing[30] using dipicolonic acid and imipenem, modified-Hodge test (MHT)[31] or three-dimensional (3D) bioassay. More often than not, these bacteria are resistant to almost all antibiotics. Knock-out or complementation assay becomes unfeasible due to unavailability of appropriate antibiotic selection marker. Therefore, alternative methods are needed to overcome this limitation.

Radiation energy was discovered more than hundred years ago, however, there has been no known research in the field of microbiology demonstrating its effect on multi-drug resistant bacteria. X-rays, gamma rays and proton particles are being used in cancer treatment to kill the cancerous cells and shrink the tumors. Proton beam radiation was also used in *E. coli* to check the effect of the high-energy beams on the bacterial morphology changes and survival rates.[32,33] However, no studies have shown the effect of radiation on the antibiotic susceptibility of the strain.

Multi-drug resistant (MDR) clinical isolates are routinely collected and maintained in Severance hospital, Seoul to keep a check on antimicrobial resistance surveillance. The hospital routinely determines the resistance mechanisms and reports carbapenem-resistant bacteria producing KPC, NDM, OXA, etc. according to the national law to try and control CREs. During one such routine analysis, a clinical isolate carbapenemase non-producing carbapenem-resistant *K. pneumoniae* YmcD1 showed strong positive results using 3D bioassay, indicating that the strain produced carbapenemase enzyme. However, no known carbapenemase genes were present in the strain as confirmed by PCR. In addition, MHT using ertapenem, imipenem and meropenem were negative. Therefore, the strain was further studied to find the cause of carbapenem resistance as well as the reason for the positive 3D bioassay. YmcD1 is a MDR strain thereby preventing us from performing transposon mediated mutagenesis due to unavailability of appropriate antibiotic selection markers. Hence radiation mediated mutagenesis using proton beam radiation was used to render the bacteria susceptible to carbapenems.

II. MATERIALS AND METHODS

1. Bacterial identification and characterization

K. pneumoniae YmcD1 clinical strain was obtained during a routine collection in Severance Hospital, Seoul. The strain was identified using Bruker MALDI Biotyper CA System (Bruker Daltonik GmbH, Bremen, Germany). Two other *K. pneumoniae* clinical isolates KPN_NDM_5026 and KPN_KPC_151 producing NDM and KPC enzymes, respectively, were also included in the study.

Four *K. pneumoniae* clinical isolates from a panel of strains maintained at our laboratory were used for this study.[34] In addition, two OmpK36 mutant strains, *OmpK36*-193::T30 and *OmpK36*-127::T30, along with parent strain MKP-103, belonging to ST258, were obtained from Manoil Lab, University of Washington, Seattle, WA, USA. These strains were used for complementation assay.

2. Antibiotic susceptibility testing

Minimum inhibitory concentration (MIC) test was performed using VITEK® 2 System (BioMérieux Marcy-l'Étoile, France) and the data were interpreted as per Clinical Laboratory Standards Institute (CLSI) guidelines.[35]

The disk diffusion assay was performed for all the strains using meropenem (10 µg), ertapenem (10 µg) and imipenem (10 µg) disks (BD BBL™ Sensi-Disk ™, Sparks, MD, USA). The zone diameters were measured and the results were interpreted as per the CLSI guidelines.[35]

3. Carbapenem modified-Hodge test

The MHT was performed as previously described.[31] Briefly, 0.5 McFarland *E. coli* ATCC 25922 was evenly spread on MacConkey plate using a cotton bud. The antibiotic disk (imipenem, ertapenem, meropenem) was placed at the center of the plate. Using a loop, the test strain was streaked from the edge of the disk to the edge of the plate. The plate was incubated for 16-18 hr at 37°C and a clover leaf appearance around the disk indicated that the strains produced carbapenemase.

4. 3D bioassay

The test strains were grown overnight in Muller-Hinton agar plate at 37°C. A 10 µl loopful of bacteria was suspended in 1 ml of distilled water and sonicated for 5 sec, with 5 sec of no sonication, for 2 min at 4°C. The sonicated cells were centrifuged at 20,000 g for 10 min at 4°C. The supernatant was collected and placed on ice. Meanwhile, fresh Muller-Hinton plates were spread with 0.5 McFarland *E. coli* ATCC 25922. The antibiotic disks were placed in the center of the plate. A slit was made 5 mm away from the edge of the antibiotic disk to the edge of the plate. Fifty microlitres of the sonicated supernatant was added into the slits such that it did not overflow. The plates were incubated overnight at 37°C. A clover leaf pattern at the intersection of the slit and the disk indicated the presence of carbapenemase enzyme.

5. Radiation mutagenesis using proton beam radiation

The bacterial strains were grown overnight in LB broth. After centrifugation, the pellets were washed re-suspended in 25 ml phosphate buffered saline (PBS) and

placed in ice throughout except during radiation. The proton beam conditions were: 20 Gy and 35 MeV. Considering the 2 mm aluminum window and 3 m air, the final energy radiating the bacteria was 20.55 MeV.

The radiated sample was serially diluted to find the appropriate dilution such that each plate has a growth of 70-80 colonies. The diluted sample was inoculated in around two hundred antibiotic free MacConkey agar plates. The following day, the plates were air-dried and replica plated using Whatman filter paper into 0.5 µg/ml meropenem containing MacConkey plates. All the plates were incubated at 37°C overnight. The colonies were manually verified, i.e., colonies that did not grow in antibiotic carrying plates were picked for further study. This procedure was repeated daily for 2-3 weeks and about 100,000 colonies were screened.

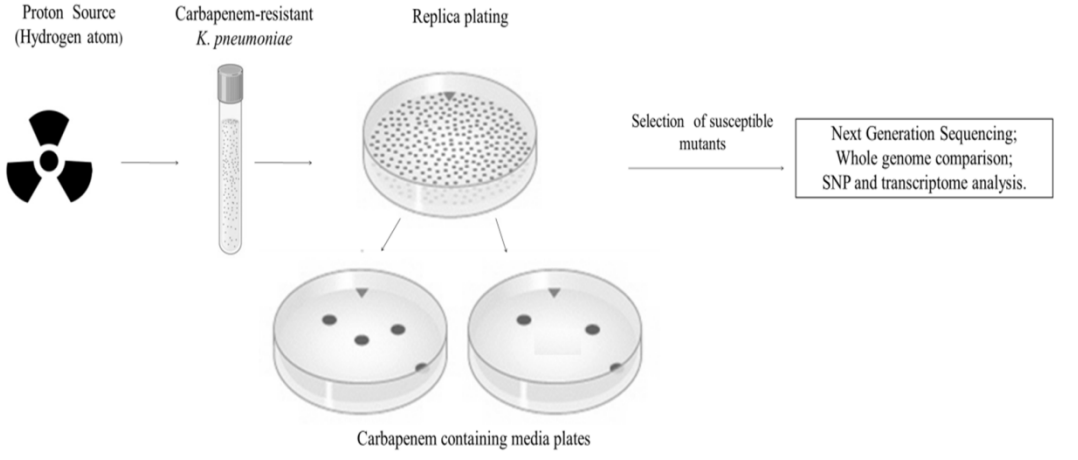

Figure: 2.1. Schematic representation of radiation-mediated mutagenesis.

6. Whole genome sequence (WGS) and data analysis

The Wizard® genomic DNA purification kit (Promega, Madison, WI) was used for DNA extraction of YmcD1 according to the manufacturer's protocol. The Qubit® dsDNA BR assay kit (Molecular Probes, Eugene, OR) was used to estimate the DNA concentration. PacBio (Pacific Biosciences, Menlo Park, CA, USA) single-molecule real time (SMRT) sequencing was carried out and the obtained WGS were annotated using RAST annotation pipeline.[36] Genome analysis was carried out using Geneious 8.1.8 (http://www.geneious.com) (Biomatters). Screening of β-lactamase genes in WGS was carried out using ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/) and further verified using NCBI BLAST.

7. Complementation assay

bla$_{\text{CMY-10}}$ gene was complemented into the susceptible mutant YmcD2 using ZpUC-19 vector (a gift from Y. Suzuki at the J. Craig Venter Institute, La Jolla, CA, USA). The primer list is provided in Table 2.1. The *bla*$_{\text{CMY-10}}$ gene was cloned into ZpUC-19 vector and transformed into DH5α *E. coli* competent cells (New England BioLabs, Ipswich, MA, USA) using heat-shock treatment. The transformants were selected on a low-salt LB agar plate containing 25 μg/ml of zeocin® (InvivoGen, San Diego, CA, USA). The plasmids were purified from the transformants using QIAprep Spin Miniprep Kit (Qiagen GmbH, Hilden, Germany) and confirmed using Sanger sequencing.

The plasmid was electroporated into the appropriate strains and the transformants were selected on 1,000 μg/ml of zeocin containing low salt LB agar and confirmed using M13F-pUC and M13R-pUC primers.

Table 2.1. List of primers used in this study

Gene	Primer name	Sequence (5'-3')
***bla*$_{CMY-10}$**	CMY10_F	TGATTACGCCAAGCTGGTAAGATACTTCGGATGAGGAGC
	CMY10_R	CGGTACCCGGGGATCTCGTCGAGGCCTGGATGG
ZpUC-19 plasmid	M13F-pUC	GTTTTCCCAGTCACGAC
	M13R-pUC	CAGGAAACAGCTATGAC

III. RESULTS

1. Carbapenem-susceptible mutant for YmcD1 obtained after radiation

Carbapenem-resistant *K. pneumoniae* YmcD1 was isolated from a patient during in-patient admission at Severance Hospital, Seoul. The strain was resistant to meropenem and ertapenem and intermediate to imipenem. The MICs for the antibiotics tested are provided in Table 2.2. Two other *K. pneumoniae* clinical isolates, KPN_NDM_5026 and KPN_KPC_151 producing NDM and KPC enzymes, respectively, were also included in the study to determine if any other carbapenem resistance related genes are present in them. All the three strains were radiated using proton beams and carbapenem-susceptible mutants were selected on meropenem containing MacConkey plates.

Radiation of KPN_NDM_5026 and KPN_KPC_151 isolates did not yield any susceptible mutants. However, susceptible mutant of YmcD1, designated as YmcD2, was obtained upon radiation and presented negative 3D bioassay results. Thus, it was confirmed that the gene responsible for the resistance had been knocked out.

Table 2.2. MIC of carbapenem-resistant strain YmcD1, radiated carbapenem-susceptible mutant YmcD2, and YmcD2 complemented with ZpUC-19 empty vector (YmcD2::ZpUC19) and vector carrying bla_{CMY-10} (YmcD2::ZpUC19_CMY10)

Strain	AM	AMC	TZP	FOX	CTX	CAZ	FEP	ATM	ETP	IPM	MEM	AMK	GM	CIP	TG	SAM*
YmcD1	≥32	≥32	≥128	≥64	≥64	≥64	8	8	≥8	2	4	16	≥16	≥4	1	6
YmcD2	≥32	≥32	≥128	≥64	2	≤1	4	≤1	≤0.5	1	0.25	16	≥16	≥4	1	6
YmcD2::ZpUC19	≥32	≥32	≥128	≥64	≤1	≤1	2	≤1	≤0.5	0.5	0.25	16	≥16	≥4	1	6
YmcD2::ZpUC19_CMY10	≥32	≥32	≥128	≥64	≥64	≥64	8	16	≥8	2	4	16	≥16	≥4	≤0.5	6

* disk diffusion (zone diameter); AM, ampicillin; AMC, amoxicillin/clavulanic acid; TZP, piperacillin/tazobactam; FOX, cefoxitin; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; ATM, aztreonam; ETP, ertapenem; IPM, imipenem; MEM, meropenem; AMK, amikacin; GM, gentamicin; TG, tigecycline; SAM, ampicillin/sulbactam;

To find the cause of resistance, whole genome sequencing was carried out for YmcD1 and its mutant YmcD2. A 9 kb nucleotide fragment was deleted from the plasmid of YmcD2 that associated with class 1 integron encoding $bla_{\text{CMY-10}}$ gene (Figure 2.2). PCR confirmed that the susceptible mutant YmcD2 indeed lacked $bla_{\text{CMY-10}}$, a class C β-lactamase gene. Whole genome analysis showed that the outer membrane porins, OmpK35 and OmpK36, were present in both the parent and susceptible mutant. Complementation of $bla_{\text{CMY-10}}$ into susceptible mutant YmcD2, designated as YmcD2::ZpUC19_CMY10, reverted the strain back to resistant phenotype (Figure 2.3).

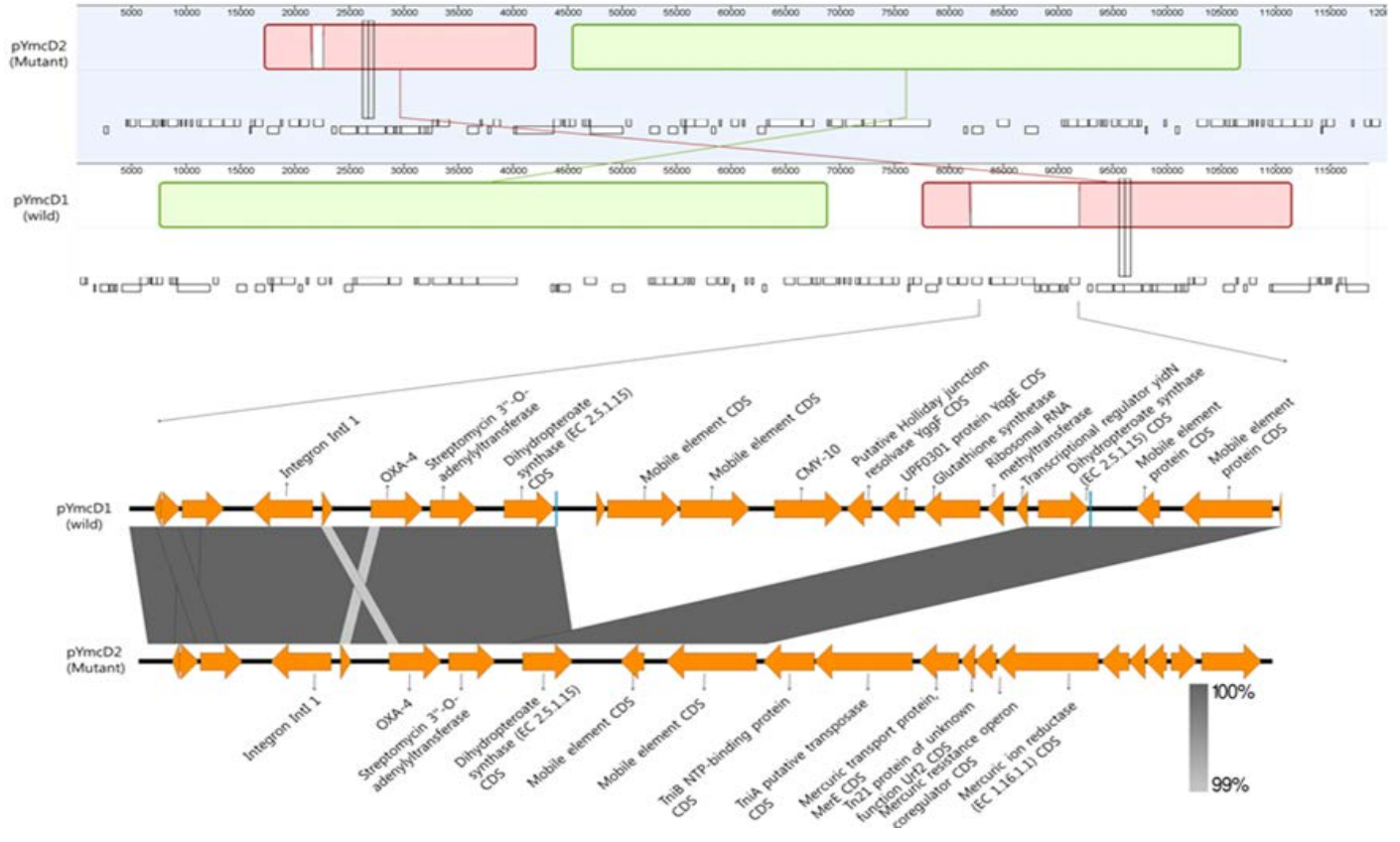

Figure 2.2. Progressive Mauve alignment of plasmid-encoding *K. pneumoniae* YmcD1 with its susceptible mutant YmcD2. The lower panel shows the excision of 9 Kb fragment encoding $bla_{\text{CMY-10}}$ indicated by thin blue vertical lines.

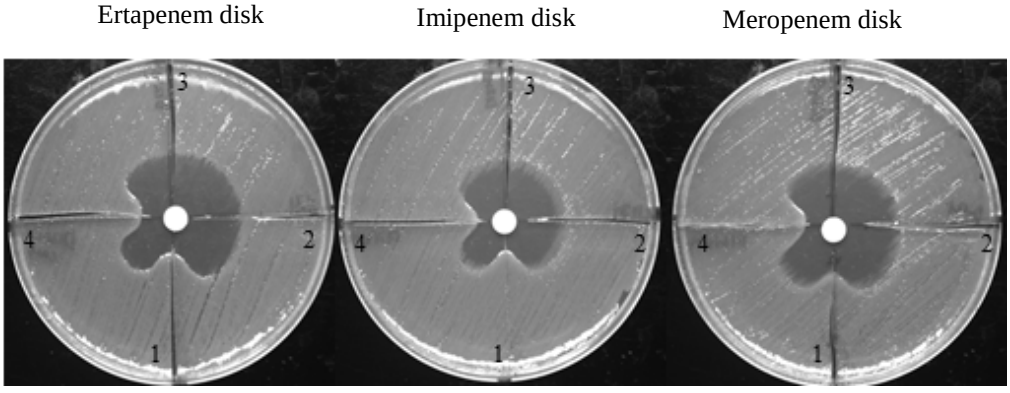

Figure 2.3. Three dimensional bioassay for YmcD1, YmcD2, and YmcD2 complemented with empty vector (YmcD2::ZpUC19) and *bla*$_{\text{CMY-10}}$ (YmcD2::ZpUC19_CMY10). The 3D bioassay was carried out using ertapenem, imipenem and meropenem disks and the samples in the slits are as follows: (1), YmcD1, (2), YmcD2, (3), YmcD2::ZpUC19, (4), YmcD2::ZpUC19_CMY10. The positive results (clover leaf structure) seen for resistant strain YmcD1 and *bla*$_{\text{CMY-10}}$-complemented carbapenem susceptible mutant YmcD2 (YmcD2::ZpUC19_CMY10) indicated that *bla*$_{\text{CMY-10}}$ was the cause of this phenotype.

2. Effect of *bla*$_{\text{CMY-10}}$ on outer membrane protein mutant *K. pneumoniae* strains

To further confirm the role of *bla*$_{\text{CMY-10}}$ in outer membrane protein mutant *K. pneumoniae* strains, *bla*$_{\text{CMY-10}}$ was complemented into *OmpK36*-193::T30 and *OmpK36*-127::T30 obtained from the parent strain MKP-103. While the parent strain lacked OmpK35 porin, the mutants lacked both OmpK35 and OmpK36 porins. The complemented strains, *OmpK36*-193::T30::ZpUC19_CMY10 and *OmpK36*-127::T30::ZpUC19_CMY10, showed increase in carbapenem MIC (Table 2.3), while the parent strain MKP-103::ZpUC19_CMY10 lacking only OmpK35 showed increased MIC for meropenem and ertapenem and not for imipenem (Table 2.3, Figure 2.4). Therefore, carbapenem resistance in *bla*$_{\text{CMY-10}}$ expressing strain is attributed to loss of both the porins.

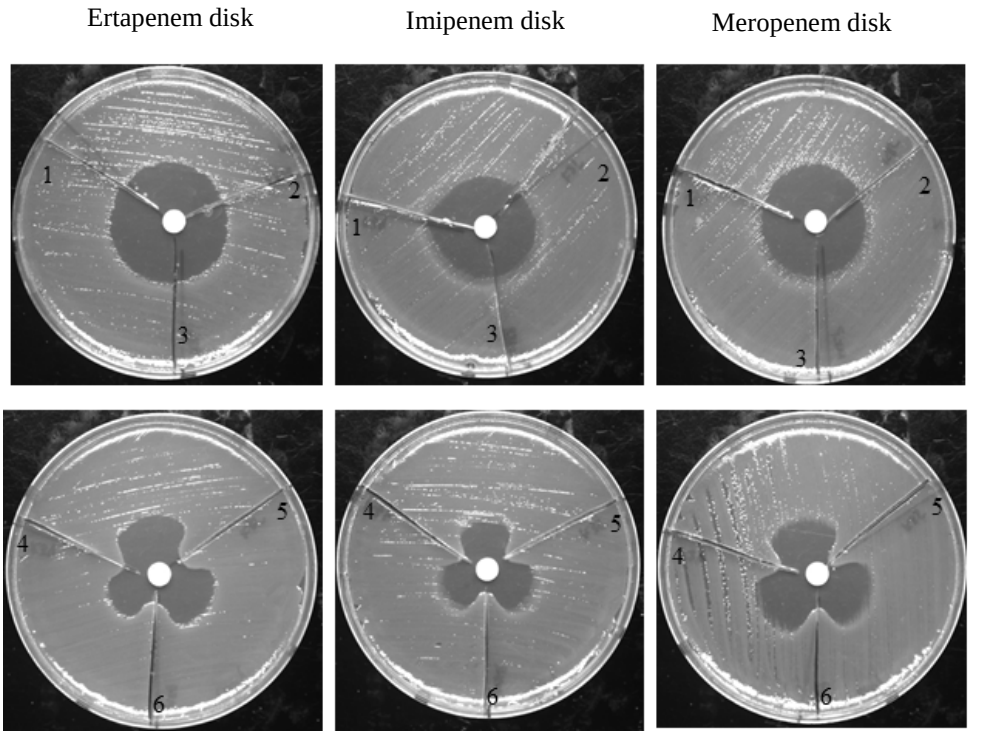

Figure 2.4. Three dimensional assay for porin mutants complemented with empty vector and *bla*$_{\text{CMY-10}}$. The 3D bioassay was carried out using ertapenem, imipenem and meropenem disks and the samples in the slits are as follows: (1), *OmpK36*-193::T30::ZpUC19, (2), *OmpK36*-127::T30::ZpUC19, (3), MKP103::ZpUC19, (4), *OmpK36*-193::T30::ZpUC19_CMY10, (5) *OmpK36*-127::T30::ZpUC19_CMY10, (6), MKP-103::ZpUC19_CMY10. The positive results are indicated by the appearance of clover leaf structure. All the three strains complemented with *bla*$_{\text{CMY-10}}$, i.e. 4, 5, and 6,show positive 3D bioassay positive thereby concluding that *bla*$_{\text{CMY-10}}$ is responsible for this phenotype.

Table 2.3. MIC and OMP profile for *K. pneumoniae* porin mutants, *OmpK36*-127::T30 and *OmpK36*-193::T30, along with their parent strain MKP103, and *K. pneumoniae* clinical strains, complemented with empty vector and bla_{CMY-10}

Strain	MIC (mg/L)			Ratio of MIC change			OmpK35	OmpK36	bla_{CMY-10}
	MEM	ETP	IPM	MEM	ETP	IPM			
OMP mutant *K. pneumoniae* strains									
MKP-103	0.25	0.5	0.094	1	1	1	−	+	−
MKP-103::ZpUC19	0.25	0.5	0.094	1	1	1	−	+	−
MKP-103::ZpUC19_CMY10	3	12	0.38	12	24	4	−	+	+
OmpK36-127::T30	0.5	1	0.19	1	1	1	−	−	−
OmpK36-127::T30::ZpUC19	0.5	1	0.19	1	1	1	−	−	−
OmpK36-127::T30::ZpUC19_CMY10	24	≥32	12	48	≥32	63	−	−	+
OmpK36-193::T30	0.5	1	0.19	1	1	1	−	−	−
OmpK36-193::T30::ZpUC19	0.5	1	0.19	1	1	1	−	−	−
OmpK36-193::T30::ZpUC19_CMY10	24	≥32	12	48	≥32	63	−	−	+
Panel strains of *K. pneumoniae* clinical strains									
YMC2011/7/B774	0.25	1.5	0.25	1	1	1	+	−	−
YMC2011/7/B774::ZpUC-19	0.25	1.5	0.25	1	1	1	+	−	−
YMC2011/7/B774::ZpUC19_CMY10	1.5	12	0.5	6	8	2	+	−	+
YMC2013/7/B3993	0.125	0.25	0.125	1	1	1	−	+	−
YMC2013/7/B3993::ZpUC-19	0.125	0.25	0.125	1	1	1	−	+	−
YMC2013/7/B3993::ZpUC19_CMY10	0.5	3	0.38	4	12	3	−	+	+
YMC2011/7/B7207	0.032	0.032	0.125	1	1	1	+	−	−
YMC2011/7/B7207::ZpUC-19	0.032	0.032	0.125	1	1	1	+	−	−
YMC2011/7/B7207::ZpUC19_CMY10	0.125	0.38	0.125	4	12	1	+	−	+
YMC2011/11/B7578	0.38	3	0.125	1	1	1	−	−	−
YMC2011/11/B7578::ZpUC-19	0.38	3	0.125	1	1	1	−	−	−
YMC2011/11/B7578::ZpUC19_CMY10	6	≥32	4	16	≥11	32	−	−	+

MEM, meropenem; ETP, ertapenem; IMP, imipenem; OMP, outer membrane protein; +, present; −, absent.
MKP-103, *OmpK36*-127::T30, and *OmpK36*-193::T30 are strains with mutated porins. These strains when complemented with empty vector form MKP-103::ZpUC19, *OmpK36*-127::T30::ZpUC19, and *OmpK36*-193::T30::ZpUC19 and when complemented with bla_{CMY-10} are designated as MKP-103::ZpUC19_CMY10, *OmpK36*-127::T30::ZpUC19_CMY10 and *OmpK36*-193::T30::ZpUC19_CMY10.
YMC2011/7/B774, YMC2013/7/B3993, YMC2011/7/B7207, and YMC2011/11/B7578 are ESBL-producing *K. pneumoniae* clinical strains susceptible to carbapenems

3. Effect of *bla*$_{CMY-10}$ in clinical *K. pneumoniae* strains

To find the MIC changes in clinical strains, *bla*$_{CMY-10}$ was complemented into four well characterized *K. pneumoniae* clinical strains obtained from a panel of strains maintained at our laboratory. Originally all the strains were ESBL-producers and susceptible to carbapenems. After complementation, the strains that lost either one of the porin did not show resistance to the carbapenems tested except for YMC2011/7/B774::ZpUC-19_CMY10 (Table 2.3). However, *K. pneumoniae* YMC2011/11/B7578::ZpUC-19_CMY10 showed increased carbapenem resistance similar to the OMP mutants *OmpK36*-193::T30 and *OmpK36*-127::T30 complemented with *bla*$_{CMY-10}$. The common similarity between these two strains was loss of both the porins, thereby proving that porin loss is the prerequisite condition.

IV. DISCUSSION

Carbapenem resistance is a serious problem worldwide and attempts are being made to find novel resistance genes and mechanisms. In this study, $bla_{\text{CMY-10}}$ was studied for its role in carbapenem resistance. A previous study proved that $bla_{\text{CMY-10}}$ hydrolyses imipenem using steady state kinetics thereby proving $bla_{\text{CMY-10}}$ to have slight carbapenemase activity.[37] In addition, $bla_{\text{CMY-10}}$-producing strains were carbapenem-susceptible. In this study, when $bla_{\text{CMY-10}}$ was complemented into *K. pneumoniae* clinical strains and porin mutants, high carbapenem resistance was observed strains lacking both the porins. The positive results of 3D bioassay due to $bla_{\text{CMY-10}}$ is irrespective of its resistance profile i.e. a carbapenem-susceptible strain carrying $bla_{\text{CMY-10}}$ will show positive 3D bioassay results. While loss of any one of the porins makes the clinical strains resistant to ertapenem, loss of both the porins is a prerequisite for carbapenem resistance in *K. pneumoniae* strains carrying $bla_{\text{CMY-10}}$.

To check the effect of radiation mediated mutagenesis on NDM and KPC producing strains, *K. pneumoniae* clinical isolates KPN_NDM_5026 and KPN_KPC_151 were radiated using proton beams. With more than 90,000 colonies screened for each isolate, the susceptible mutants could not be found. Less fit organisms have a higher probability to acquire resistance along with fitness cost for plasmid carriage.[38] Hence it can be a possibility that exposure to radiation killed the bacteria that lost the KPC or NDM genes and the other bacteria survived.

Proton beams radiation is a novel approach that has never been used to alter the antibiotic susceptibility of MDR strains to date. The drawbacks of this method are its labor intensiveness and lower efficiency of bacterial transformation. In addition, the screening of the colonies had to be carried out within a week after radiation due to rapid decrease in viable bacteria. Furthermore, additional training and education is required with regards to radiation exposure safety measures. The amount of radiation emitted from the sample after radiation was also measured so that it was in the acceptable range to be transferred out of the radiation facility.

V. CONCLUSION

This study was the first to introduce radiation-mediated mutagenesis using proton beam radiation for gene knockout. Radiation mediated mutagenesis can be used knock out genes in multi-drug resistant strains wherein the use of resistance gene markers for mutant selection is unavailable. $bla_{\text{CMY-10}}$ was found to be the causative gene for positive 3D bioassay though it is an AmpC β-lactamase. This study is also the first to report the loss of both OmpK35 and OmpK36 as prerequisite for complete carbapenem resistance in *K. pneumoniae* strains carrying $bla_{\text{CMY-10}}$.

CHAPTER III

Carbapenem resistance reversibility in *K. pneumoniae* strains obtained *in-vivo* and *in-vitro*

Carbapenem resistance reversibility in *K. pneumoniae* strains obtained *in-vivo* and *in-vitro*

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I. INTRODUCTION

Antibiotic resistance is a global health problem and deaths due to multi-drug resistant bacteria is expected to reach 300 million by the year 2050.[39] In 2014, the number of deaths due to antimicrobial resistance infections in Europe and US alone was approximately 50,000. This is a major setback in the health sector especially in this era of advanced technology and development. *Klebsiella pneumoniae* is an important opportunistic pathogen which is resistant to almost all classes of antibiotics currently available and causes nosocomial and community acquired infections.[40] Carbapenems are used as antibiotics of last resort to treat *Enterobacteriaceae* producing ESBLs. However, there has been an exponential rise in the carbapenem-resistant *K. pneumoniae* since its first report in 1996.[41] It is worrisome to see the emergence of carbapenem-resistant bacteria as there is limited availability of antimicrobial agents for treatment. Resistance mechanisms may involve modifications to porins, up-regulation of efflux pumps, ESBLs accompanied by porin loss, hyper-production of AmpC β-lactamase, and carbapenemase production.[11]

The potential of the bacteria to acquire antimicrobial resistance within the host is illustrated in Figure 3.1.[42] While inside the host, when the bacteria are exposed to antibiotics, the susceptible isolate is usually killed. However, some of them may evade killing by transforming themselves into resistant isolates. Such bacteria have a relatively high fitness cost compared to susceptible strains and resistant strains turn susceptible when exposure to antibiotics end. Moreover, these resistant strains can remain resistant by inheriting compensatory mutations that come with no associated fitness cost. Alternatively, some strains can switch the resistance on or off (adaptability) in the presence of antibiotics and thereby lowering their fitness cost. Such strains pose a high risk to public health.

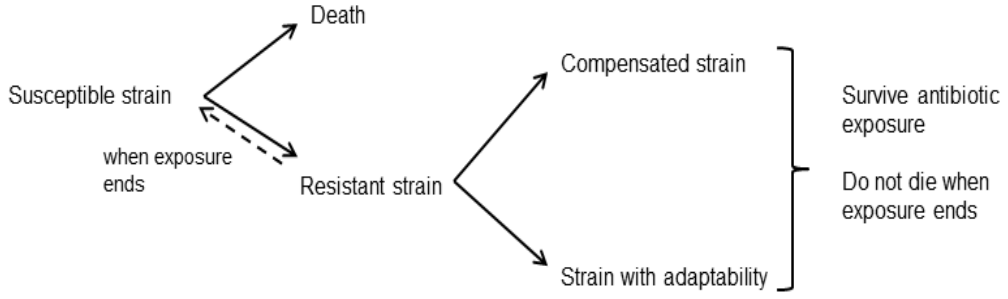

Figure 3.1. ***In-vivo*** **adaptive potential of bacteria during antibiotic exposure**. (adapted from X. Didelot *et al*. Nature reviews 2016 14:156)[42]. When a susceptible bacterial strain is exposed to an antibiotic, it is highly likely to be killed, but may occasionally survive by evolving into a resistant strain. Fitness cost due to resistance is usually high, so that resistant strains usually disappear when not exposed to the antibiotic. However, resistant strains can evolve compensatory mutations so that they remain resistant without the associated fitness cost. Such compensated strains pose a serious danger to public health, because they do not disappear as a result of antibiotic disuse. Alternatively, strains may evolve adaptability, enabling them to quickly switch resistance on or off and therefore avoid the associated fitness cost, presenting a similar risk to public health as that presented by compensated strains.

In this study, two *K. pneumoniae* strains were collected retrospectively from a patient before and after meropenem treatment along with an *in-vitro* generated meropenem-resistant mutant. Using whole genome, transcriptome, and functional network analysis, the putative candidate genes to revert the resistant strain to

susceptibility were found. This study provides an insight into uncharacterized resistance mechanisms that aid in meropenem susceptibility.

II. MATERIALS AND METHODS

1. Bacterial selection and identification

Two *K. pneumoniae* isolates K26 and K56 used in this study were selected from a collection of bacteria maintained in the laboratory of Severance Hospital, Seoul, South Korea. The strains were from a single patient who had undergone meropenem treatment. K26 was collected before while K56 was collected after the meropenem administration. The strains were identified using MALDI-TOF MS Biotyper CA System (Bruker Daltonik GmbH). The clonal relatedness was determined using pulsed-field gel electrophoresis (PFGE) as previously described.[43]

2. Antibiotic susceptibility determination

The minimum inhibitory concentration (MIC) of meropenem was determined by using E-test. *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were used as control strains as recommended by Clinical and Laboratory Standards Institute (CLSI) guidelines. The results were confirmed using three independent experiments.

3. Mutant generation acquiring meropenem resistance *in-vitro*

The carbapenem-susceptible *K. pneumoniae* strain K26 was used as the parent strain to obtain in-vitro meropenem mutants. The strain was initially cultured on an antibiotic free MacConkey agar and incubated at 37°C. Subsequently, the strain was serially grown in plates containing different meropenem concentrations i.e. 0.38, 0.5, 1, 2, 4, 8, and 16 µg/ml with overnight incubation at 37°C over a span of two weeks. Mutant strains were collected at all the concentrations, however, strain designated as K26M, obtained at the concentration of 16 µg/ml of meropenem, was selected for further analysis.

The strain K26M was used for reversion analysis by serially growing the strains in antibiotic free MacConkey plates. The strains were cultured repeatedly for 15 days and disk diffusion using meropenem disk was carried out before each passage.

4. DNA extraction, sequencing and analysis

The DNA was extracted from all the strains using Wizard® genomic DNA purification kit (Promega, Madison, WI) according to the manufacturer's protocol. The DNA concentration was estimated using Qubit® dsDNA BR assay kit (Molecular Probes, Eugene, OR) and library preparation was carried out using IonXpress™ Plus fragment library kit (Life technologies, Carlsbad, CA, USA). Whole genome sequencing was carried out on a 318 chip v2 using the Ion Torrent PGM™ system and Ion PGM™ sequencing 400 Kit (Life technologies).

K. pneumoniae HS11286 was used as the reference genome and variant calling was performed using Breseq program.[44]

5. RNA extraction, sequencing and analysis

RNA was isolated by growing the strains to the logarithmic phase in high osmolarity LB broth at 37°C and extracting the RNA using RNeasy® mini kit (Qiagen GmbH, Hilden, Germany). On-column DNA digestion was carried out using RNase-free DNase Kit (Invitrogen, Carlsbad, CA, USA). RNA concentration was measured using a Nanodrop™ spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Library preparation was carried out using TruSeq Stranded total RNA Library Preparation Kit (Illumina, San Diego, CA, USA). Illumina NextSeq 500 sequencer (Illumina, San Diego, CA, USA) was used to carry out RNA sequencing. The raw reads were aligned using STAR aligner[45] and differentially expressed genes were observed using DeSeq2 program.[46]

RNA was sequenced and analyzed for the complemented strains K56::ZpUC19-*KPHS_33600* and K26M::ZpUC19-*KPHS_46730*. Three sets of RNA sequences were generated for all the strains. KlebNet was used to find the neighboring genes related to these candidates.

6. Functional gene network preparation

'KlebNet' , a functional gene network, was constructed by our collaborators to further short-list the selected differentially expressed genes obtained after DeSeq2

program. It is available for public use at http://www.inetbio.org/klebnet/. *K. pneumoniae* HS11286 was used as reference strain for the network construction.

7. Complementation assay

The final putative candidate genes mutated in K56 and K26M were complemented into the respective strains using ZpUC-19 plasmid (pUC-19 plasmid carrying zeocin marker).[47] This plasmid was a gift from Y. Suzuki at the J. Craig Venter Institute, La Jolla, CA, USA. The primers used to amplify the genes are provided in Table 3.1. All the gene fragments were amplified using TaKaRa Ex Taq™ (Takara Bio Co., Ltd, Shiga, Japan) using the susceptible strain K26 as the template. The plasmid and the fragments were cut with appropriate restriction enzymes and purified using QIAquick® PCR purification kit (Qiagen GmbH, Hilden, Germany). The inserts were ligated to ZpUC-19 plasmid and the plasmid was transformed into DH5α *E. coli* competent cells (New England BioLabs, Ipswich, MA, USA) using heat-shock treatment. The transformants were selected on a low-salt LB agar plate containing 25 μg/ml of zeocin® (InvivoGen, San Diego, CA, USA). The plasmids were purified from the transformants and confirmed using Sanger sequencing.

The plasmids were also introduced into electrocompetent K56 and/or K26M as previously described[48] and the transformed cells were recovered with low-salt LB broth (Sigma Aldrich, St. Louis, MO, USA) and the cells were selected on a low-salt

LB agar plates carry 1,000 μg/ml of zeocin. The complemented strains were confirmed using M13F-pUC and M13R-pUC primers.

Table 3.1. List of primers used in this study

Gene	Primer name	Sequence (5'-3')
KPHS_33470	PTS_F	TAA GCA GAATTC CAAGGTCAGTCATCGCCATGC
	33470_R	TGC TTA GGATCC CTGTTGATTAAGTCTTAAAG
KPHS_33480	33480_F	TGATTACGCCAAGCTAAGAAATGCGGGATACGTAGAGG
	33480_R	CGGTACCCGGGGATCCTCACCTGTTAGTCCAGTTCG
KPHS_33490	33490_F	TAA GCA GAATTC CACTTTAAGACTTAATCAAC
	PTS_R	TGC TTA GGATCC GAAGGTCATTGCTTTGCGTTTC
KPHS_33510	33510_F	TAA GCA GAATTC CCACGGCTATATTTCCCGCCTA
	33510_R	TGC TTA GGATCC GCTTAACCGGCGAAATGGC
KPHS_33590	33590_F	TAA GCA GAATTC TTGGTAGGCGTTAACGATCCA
	33590_R	TGC TTA GGATCC CGATCGCGACGTAGCGCC
KPHS_33600	33600_F	TAA GCA GAATTC ACCTCGTCGTAACTGTT
	33600_R	TGC TTA GGATCC TTCTGACGCTGAAAACG
KPHS_35510	35510_F	TAA GCA GAATTC TATTCAGGGCATCGACAG
	35510_R	TGC TTA GGATCC TATTTCAACATGATTGGTC
KPHS_11800	11800_F	TAA GCA GAATTC CCTCATACCTCATCCATTCTGCC
	11800_R	TGC TTA GGATCC GGATAACGCCATGCGCCAAT
KPHS_33520	33520_F	TAA GCA GAATTC CGCGGGTGATGCCGGAGAGTATT
	33520_R	TGC TTA GGATCC GCAGCGAAATCCTCTGGAGCC
KPHS_33460	33460_F	TGATTACGCCAAGCTGTTAATCACGTATCGTCTGCG
	33460_R	CGGTACCCGGGGATCAACTACCTGCGTAGCCACG
KPHS_33500	33500_F	TAA GCA CTGCAG CATTCCGATGGTGGTGTC
	33500_R	TGC TTA GAATTC TCCGCTGCTAACGGGAATA
KPHS_46730	46730_F	TAA GCA CTGCAG CTGGTCATTACGCTGATG
	46730_R	TGC TTA GAATTC TCGCACTGTTCGGCAATC
KPHS_33610	33610_F	TGATTACGCCAAGCTCCGCTGTCAGCAAGATGC
	33610_R	CGGTACCCGGGGATCAATCCGCAGCTTGCGGGC
OmpK36	37010_F	TAA GCA AAGCTT GATATGCTGTCGTCTATCGC
	37010_R	TGC TTA GGATCC CAAGAGTATACCAGCGAGG
ZpUC-19 plasmid	M13F-pUC	GTTTTCCCAGTCACGAC
	M13R-pUC	CAGGAAACAGCTATGAC

8. Growth assay experiment

The complemented strains along with parents with blank plasmids were grown in 5 ml LB broth at 37°C with continuous shaking. The following day, the strains were inoculated into fresh LB broth with and without meropenem (1 µg/ml, 2 µg/ml, 4 µg/ml, and 8 µg/ml) and OD_{600} was measured using U.V. spectrophotometer at 0, 1, 2, 3, 4, 5, 6, 8, 12, and 24 hr. The values were plotted using GraphPad Prism 5.01 for Windows (GraphPad Software Inc., San Diego, CA, USA). The experiment was carried out in triplicates.

9. Virulence study using *Galleria mellonella* larvae

G. mellonella larvae were purchased from SWorm Ltd, Daejeon, South Korea and were used within 5 days of receipt. The bacterial strains were grown in low-salt LB broth for 3 hr and centrifuged at 4,000 g for 20 min at room temperature. The obtained pellets were washed once and re-suspended in PBS buffer to obtain a known concentration. The larvae were injected with 10 µl of the bacterial cells (OD_{600} 1.0) into the last left proleg using a Hamilton syringe and incubated at 37°C inside the petri-plate. Control strains were maintained by injecting 10 µl of PBS buffer. Survival of the larvae were recorded every 24 hr up to 4 days.

Similarly, 4 hr post-infection the larvae were injected with a single dose of 60 mg/kg of meropenem and the survival rate was monitored every 24 hrs up to 4 days.

III. RESULTS

1. Bacterial strain selection and characterization

The two selected *K. pneumoniae* strains were isolated from a single patient before (K26) and after (K56) receiving meropenem treatment. The strains were isogenic as determined by PFGE and differed only in their susceptibility to carbapenems. The MIC and β-lactamase genes present in both the strains are shown in Table 3.2.1 and 3.2.2. In addition, to examine the effects of antibiotic stress on the adaptability of the isolates, strain K26 was grown *in-vitro* in increasing concentrations of meropenem until MIC of 32 μg/ml (K26M) was acquired. When K26M strain was grown in antibiotic-free MacConkey agar plates repeatedly up to 15 days, the resistance phenotype remained irreversible. No changes in the disk diffusion pattern was observed.

Table 3.2.1. MIC of the strains used in this study

Strain	AM	AMC	TZP	FOX	CTX	CAZ	FEP	ATM	ETP	IPM	MEM	AMK	CIP	TG
K26 (susceptible)	≥32	≥32	≥128	≥64	≥64	≥64	≥64	≥64	4	1	0.5	≥64	≥4	2
K56 (in-vivo resistant)	≥32	≥32	≥128	≥64	≥64	≥64	≥64	≥64	≥8	≥16	≥16	≥64	≥4	2
K26M (in-vitro resistant)	≥32	≥32	≥128	≥64	≥64	≥64	≥64	≥64	≥8	≥16	≥16	≥64	≥4	2

AM, ampicillin; AMC, amoxicillin/clavulanic acid; TZP, piperacillin/tazobactam; FOX, cefoxitin; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; ATM, aztreonam; ETP, ertapenem; IPM, imipenem; MEM, meropenem; AMK, amikacin; CIP, ciprofloxacin; TG, tigecycline.

Table 3.2.2. β-lactamase genes and outer membrane protein profile using whole genome analysis

Strain	β-Lactamase genes	Mutation of porin genes	
		OmpK35	**OmpK36**
K26	$bla_{\text{SHV-12}}$, $bla_{\text{DHA-1}}$, $bla_{\text{LEN-11}}$	54T deletion	NM
K56	$bla_{\text{SHV-12}}$, $bla_{\text{DHA-1}}$, $bla_{\text{LEN-11}}$	54T deletion	Transposon insertion
K26M	$bla_{\text{SHV-12}}$, $bla_{\text{DHA-1}}$, $bla_{\text{LEN-11}}$	54T deletion	Stop codon at 74^{th} position

NM, no mutation.

2. Candidates confirmed after complementation

Genes that were mutated in K56 and K26M strains but not altered in K26 were found using WGS. Among them, only the differentially expressed genes were determined using transcriptome data and analyzed further. A total of 78 genes were finalized and further analyzed using KlebNet. Finally, 14 putative candidates were short-listed for the complementation assay with $p<0.05$ (Table 3.3). Among them, 12 genes were mutated in K56, one gene in K26M, and one mutated gene (*KPHS_46730*) was common among both the resistant strains. Upon complementation, two positive candidates were obtained, namely, K56::ZpUC19-*KPHS_33600* and K26M::ZpUC19-*KPHS_46730* (Figure 3.2). The strain K56::ZpUC19-*KPHS_33600* is *KPHS_33600* gene complemented into in-vivo resistant strain K56, and K26M::ZpUC19-*KPHS_46730* is *KPHS_46730* gene complemented into in-vitro resistant strain K26M. Although *KPHS_46730* mutation was common in both strains, partial meropenem susceptibility restoration was observed in K26M strain alone.

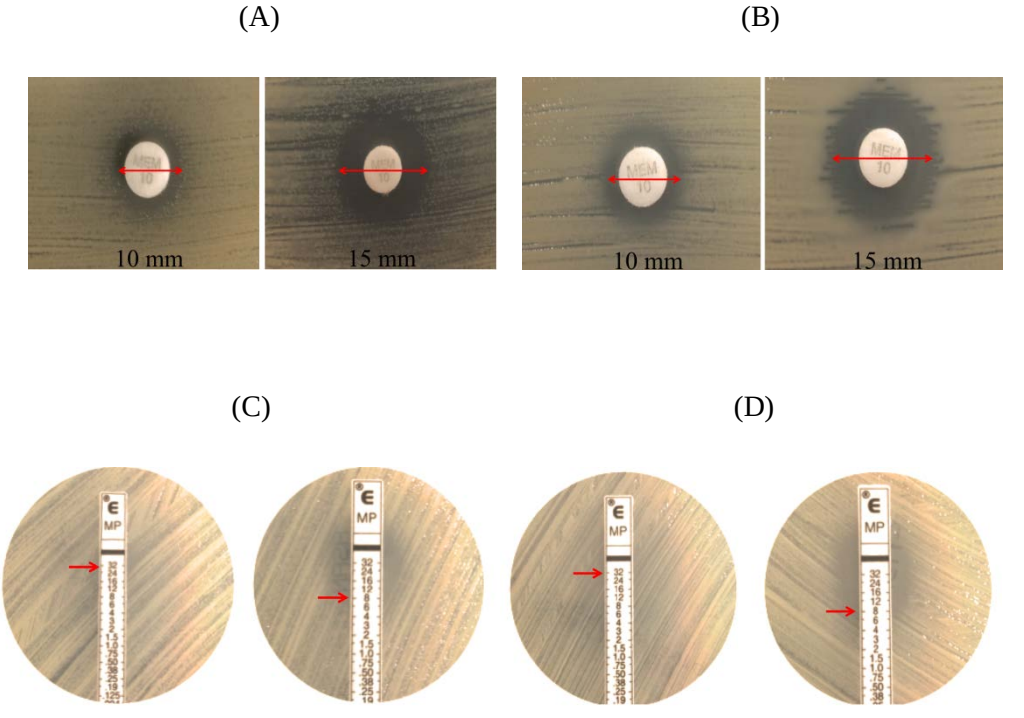

Figure 3.2. Antibiotic susceptibility testing of candidate genes using meropenem disks and meropenem E-test strips. (A), The zone diameter increased from 10 mm (in-vivo resistant strain K56) to 15 mm when K56 was complemented with *KPHS_33600*, (B), Increased zone diameter (15 mm) when in-vitro resistant strain K26M was complemented with *KPHS_46730*, (C) MIC of K56::ZpUC19 and K56::ZpUC19-*KPHS_33600* are 32 µg/ml and 8 µg/ml respectively as indicated using red arrows, (D), Similarly, MIC of K26M::ZpUC19 and K26M::ZpUC19-*KPHS_46730* are 32 µg/ml and 8 µg/ml, respectively. Upon complementation, a 4-fold reduction in MIC of both the resistant strains can be seen.

Table 3.3. Final candidate genes used for experimental validation

Gene	Mutation	Log_2 fold change	Annotation
KPHS_11800	(A)5>6 (131/183 nt)	-0.0528	50S ribosomal protein L36
KPHS_33460	Whole Gene Deletion	-0.7504	putative transmembrane protein
KPHS_33470	Whole Gene Deletion	-3.2255	PTS enzyme IIAB, mannose-specific
KPHS_33480	Whole Gene Deletion	-4.1758	PTS enzyme IIC, mannose-specific
KPHS_33490	Whole Gene Deletion	-3.2904	mannose-specific PTS system protein IID
KPHS_33500	Whole Gene Deletion	-2.3410	hypothetical protein
KPHS_33510	Whole Gene Deletion	-4.1492	hypothetical protein
KPHS_33520	Whole Gene Deletion	-4.3252	ribosomal RNA large subunit methyltransferase A
KPHS_33590	Whole Gene Deletion	-6.9129	IclR family transcriptional regulator
KPHS_33600	Whole Gene Deletion	-3.9602	putative transport protein
KPHS_33610	Whole Gene Deletion	-0.5490	heat shock protein HtpX
KPHS_35510	Asp297Glu, Gln303Lys, 2bp>GC (909-910/1005 nt)	0.1130	uridine diphosphate galacturonate 4-epimerase
KPHS_46730	(T)5>6 (763/891 nt)	0.0639	alpha-dehydro-beta-deoxy-D-glucarate aldolase
KPHS_37010[#]	Tyr74*	-0.8304	OmpK36 porin
KPHS_46730[#]	(T)5>6 (763/891 nt)	-0.1587	alpha-dehydro-beta-deoxy-D-glucarate aldolase

[#] in-vitro candidates

3. Effect of outer membrane protein OmpK36 complementation

Porin loss is one of the well known mechanism studied for carbapenem resistance acqusition in *K. pneumoniae*. *KPHS_37010*, annotated as OmpK36 porin, was among the short-listed candidate gene using KlebNet for K26M strain. To determine whether OmpK36 expression decreases meropenem MIC, the gene was complemented into both the resistant strains K56 and K26M. Interestingly, there was no difference observed in the MIC values in both the strains (data not shown), thereby hinting that OmpK36 loss may not play a role in meropenem resistance in non-carbapenemase-producing *K .pneumoniae* strains.

4. Characterization of the candidates

A. *KPHS_33600* in K56

KPHS_33600 was annotated as putative transport protein using *K. pneumoniae* HS11286 as a reference. BLASTP search showed that this gene was commonly annotated as MFS transporter, which is a family of efflux pumps. Due to lack of literature on this transporter, KlebNet was used to find the neighboring genes associated with *KPHS_33600* (Figure 3.3). RNA sequencing was carried out for both K56::ZpUC19 and K56::ZpUC19-*KPHS_33600* to find the differentially expressed genes. The expression levels of these genes were observed before and after complementation i.e. K56 in comparison with K26, and K56::ZpUC19-*KPHS_33600* in comparison with K56::ZpUC19 (Figure 3.4, Table 3.4). Genes which are notably up- or down-regulated after complementation have been included in the table.

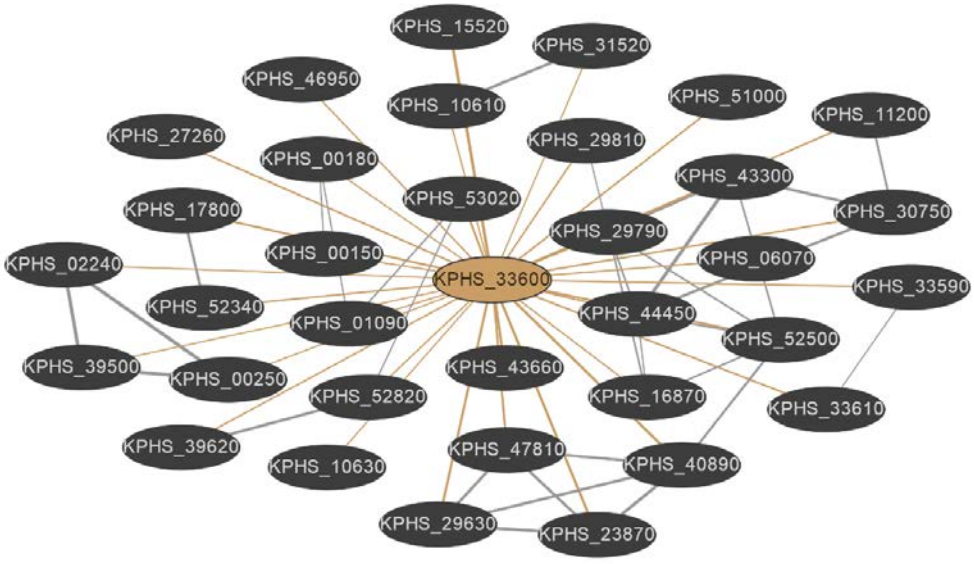

Figure 3.3. Neighboring genes of *KPHS_33600* obtained from KlebNet.

Table 3.4. List of differentially expressed neighbors of *KPHS_33600* along with annotations

Genes	Annotation
KPHS_30750	Putative sugar transport protein
KPHS_27260	Auxiliary transport protein, membrane fusion protein (MFP) family
KPHS_39620	GTPase Era
KPHS_46950	Putative periplasmic protein
KPHS_06070	HlyD family secretion protein
KPHS_00180	Stress response kinase A
KPHS_02240	DNA-binding response regulator in two-component regulatory system with ZraS
KPHS_10630	Putative permease

Note: The p-value for all the genes is <0.05

KPHS_27260 and *KPHS_06070* showed more than two-fold down-regulation in its expression. They belong to membrane fusion protein family and HlyD family secretion protein, respectively. These families of protein form an integral part of efflux pump transporter specifically acting as periplasmic adaptors. BLASTP search of KPHS_06070 showed 95% sequence identity with HlyD family protein EmrA, which is a part of the EmrAB-TolC efflux pump belonging to major facilitator superfamily.[49] Therefore, down-regulation of EmrAB-TolC efflux pump might have lead to partial meropenem susceptibility.

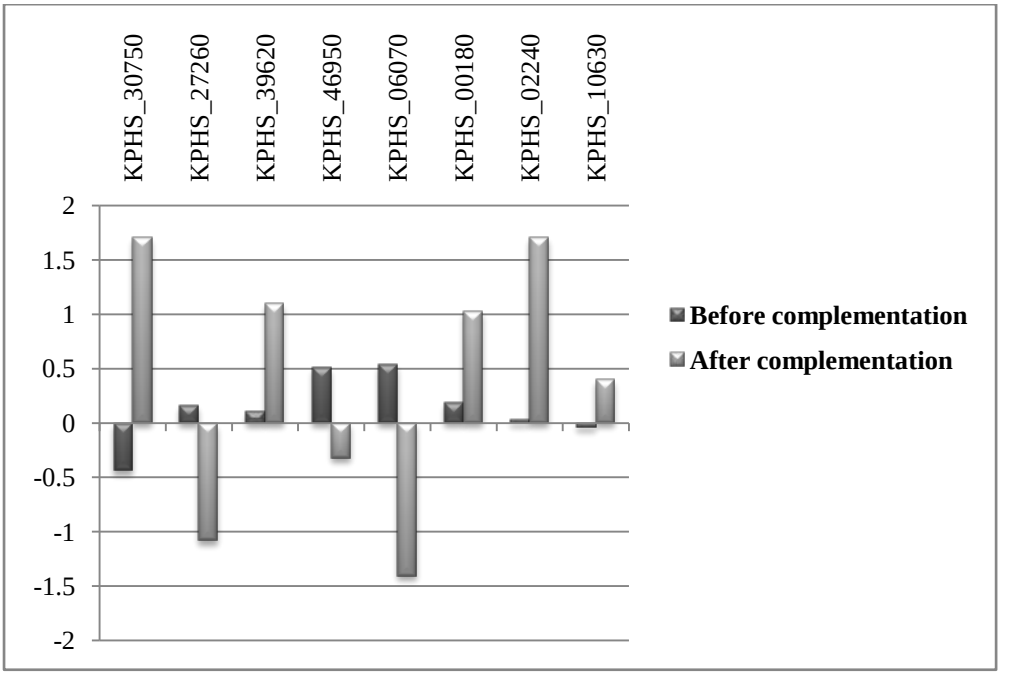

Figure 3.4. Neighboring genes of *KPHS_33600* differentially expressed in in-vivo carbapenem-resistant K56 strain before and after complementation with *KPHS_33600*. *KPHS_27260* and *KPHS_06070* show more than two fold decrease in their expression.

B. *KPHS_46730* in K26M

KPHS_46730, also known as *garL*, catalyzes the conversion of 2-dehydro-4-deoxy-D-glucarate to pyruvate and tartronate semialdehyde. Pyruvate enters the TCA cycle by converting to acetyl CoA using pyruvate dehydrogenase complex. Figure 3.5 shows the TCA cycle products and the enzymes that catalyze their conversions.

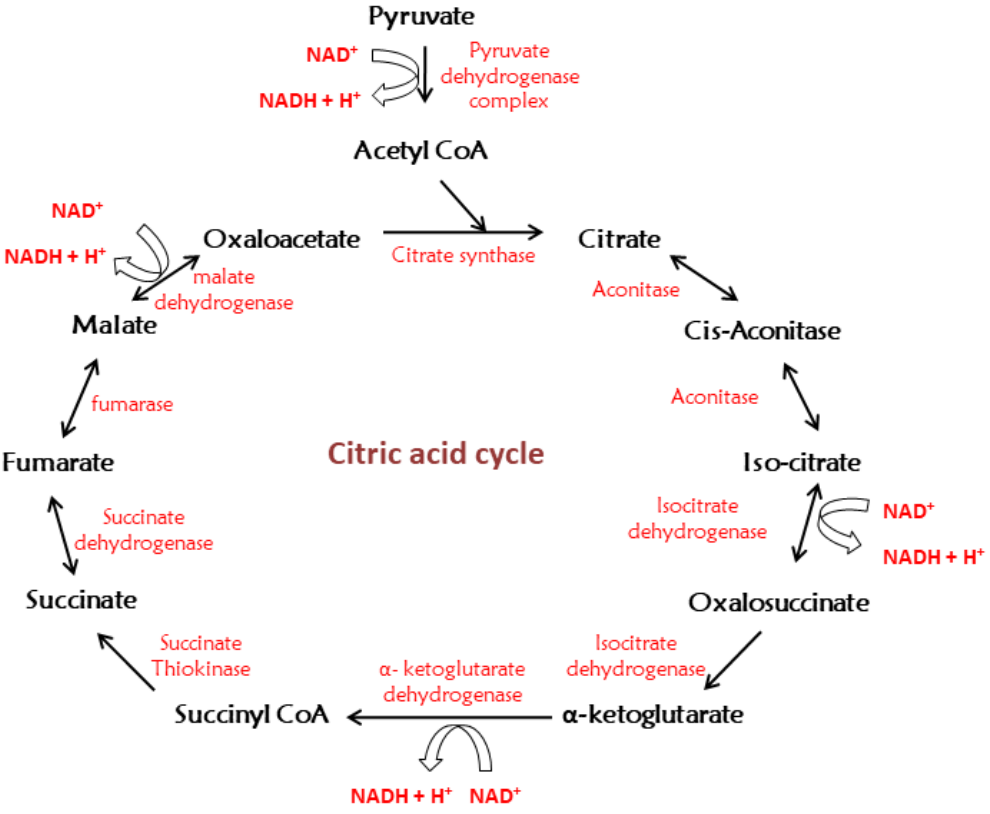

Figure 3.5. Citric acid cycle representing the enzymes and product of various reactions.

TCA cycle has a role in bacterial cell death following primary drug interactions as described in a previous study.[50] The study showed that the NADH produced by the TCA cycle is important for bacterial death. The higher the number of NADH produced, greater the rate of bacterial death (Figure 3.6). Hence, the gene expression levels of enzymes responsible for conversion of the TCA cycle products were checked manually in K26M::ZpUC19-*KPHS_46730* strain. There was significant two-fold up-regulation of citrate synthase (*KPHS_12970*, *KPHS_15630)* and aconitase (*KPHS_08450*, *KPHS_21790*) genes. Moderate down-regulation of iso-citrate dehydrogenase was observed except it was not significant ($p>0.05$). However, genes for the conversion of α-ketoglutarate to succinyl-CoA were down-regulated by greater than two folds (*KPHS_15690*, *KPHS_08400*, *KPHS_15700*). Therefore, K26M::ZpUC19-*KPHS_46730* strain produced only one NADH after complementation thus bringing about partial meropenem susceptibility.

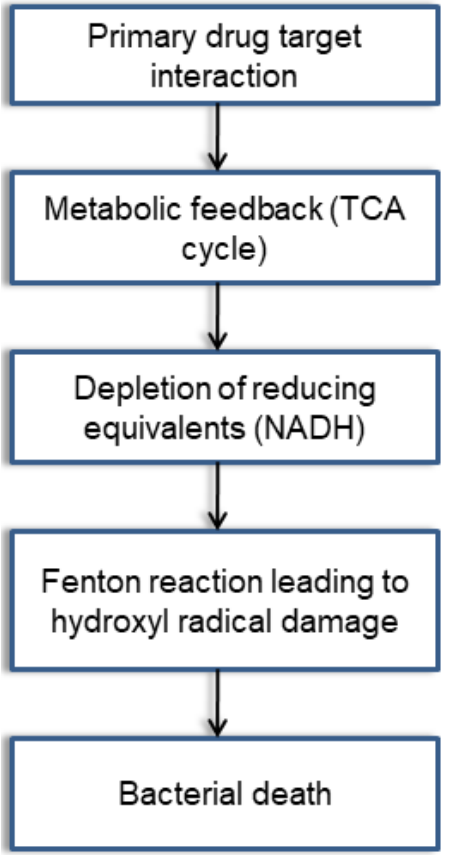

Figure 3.6. Schematic representation of hypothesis for partial killing of *K. pneumoniae* by meropenem after *garL* complementation. (adapted from Kohanski M.A. *et al* Cell 130, 797–810, September, 2007)[50]. The primary drug-target interactions (β-lactam with penicillin binding proteins) stimulate oxidation of NADH via the electron transport chain (ETC) that is dependent on the TCA cycle. Hyper-activation of the ETC stimulates superoxide formation which damages iron-sulfur clusters, making ferrous iron available for oxidation using Fenton reaction. This leads to hydroxyl radical formation, and the hydroxyl radicals damage DNA, proteins, and lipids, which result in cell death.

5. Alteration in bacterial fitness

Growth assay was carried out to determine the bacterial fitness following gene complementation. In the antibiotic-free media, no difference in bacterial growth was observed between parent and complemented strains (Figure 3.7). With exposure to 1 μg/ml and 2 μg/ml of meropenem, a significant lag in growth was observed for K56::ZpUC19-*KPHS_33600* and K26M::ZpUC19-*KPHS_46730* compared to their parent strains (Figure 3.8 and Figure 3.9). Meropenem concentration was gradually increased up to 4 μg/ml and 8 μg/ml. At 8 μg/ml, all strains failed to grow in the presence of meropenem until 24 hr. However, after 48 hr, growth was observed in resistant strains carrying empty vectors, i.e., K56::ZpUC19 and K26M::ZpUC19 strains (Figure 3.9). Overall, there was no difference in bacterial fitness due to complementation.

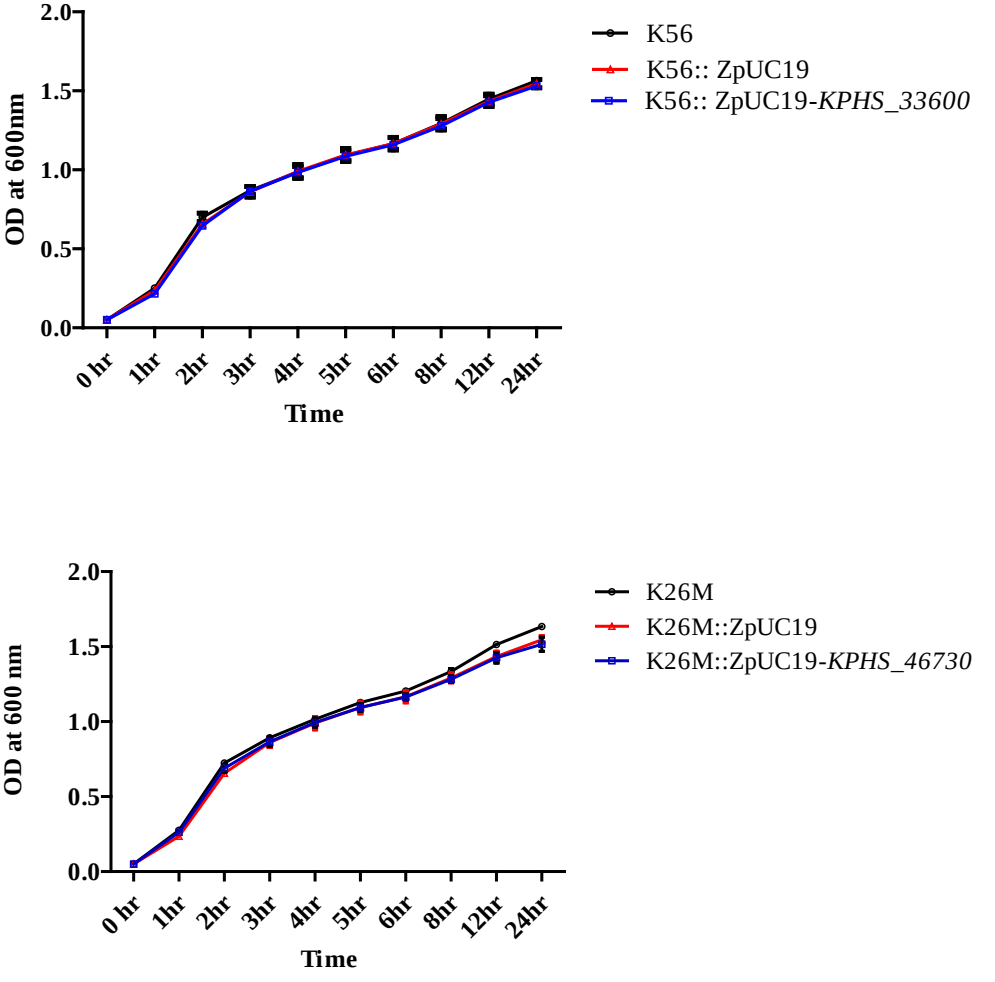

Figure 3.7. Growth assay using LB broth without meropenem. Growth assay was carried out in LB broth for the two complemented strains K56::ZpUC19-*KPHS_33600* and K26M::ZpUC19-*KPHS_46730* along with their parent strains K56 and K26M with and without ZpUC-19 empty vector, respectively. There was no difference between the resistant strains and complemented strains (both in-vivo and in-vitro).

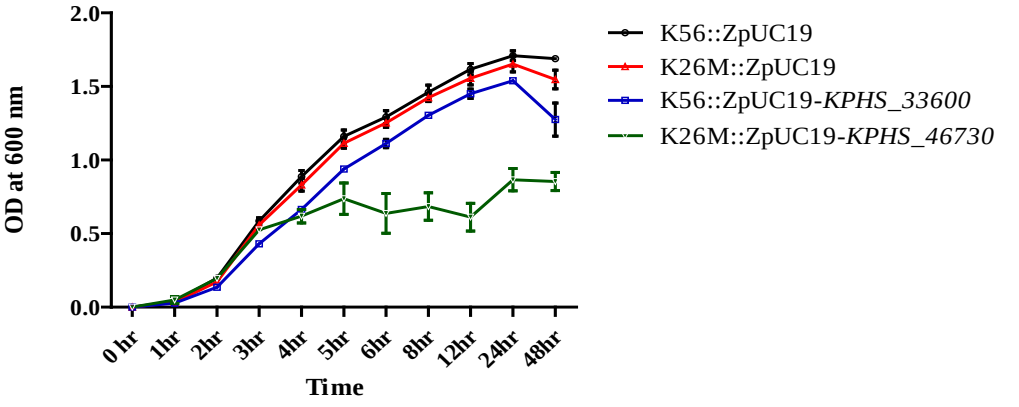

Figure 3.8. Growth assay in 1 μg/ml of meropenem. Growth assay measured for positive candidates and their respective resistant strains in the presence of 1 μg/ml of meropenem.

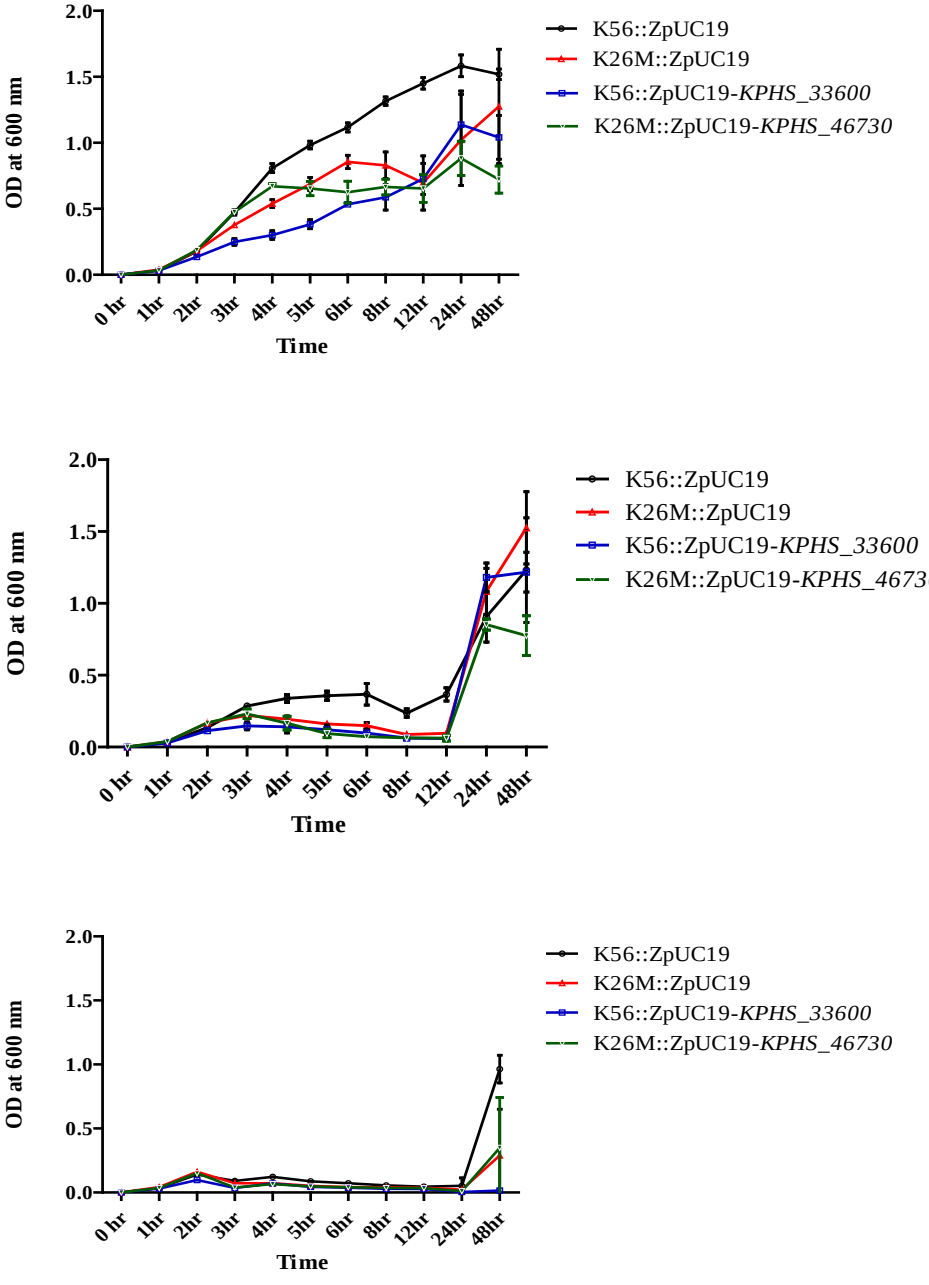

Figure 3.9. Growth assay in increasing concentrations of meropenem. The bacterial growth was measured for positive candidates and their respective resistant strains in the presence of 2, 4 and 8 μg/ml of meropenem (top to bottom).

6. *Galleria mellonella* larvae: An ideal insect model to detect virulence in *K. pneumoniae*

Studies have shown that virulence of *K. pneumoniae* in *G. mellonella* larvae can replicate that in mice model.[51] *G. mellonella* can be incubated at 37°C, mimicking human body temperatures, thus making it an ideal for bacterial infection. Therefore, *G. mellonella* larvae were used in this study to investigate the virulence of the complemented strains.

Larvae infected with K56::ZpUC19-*KPHS_33600* died within 48 hr of infection unlike K56::ZpUC19-infected larvae where 30% of the larvae survived after 96 hr (Figure 3.10A). K26M::ZpUC19-*KPHS_46730* and K26M::ZpUC19-infected larvae showed 40% and 15% survival rate after 4 days, respectively (Figure 3.10B). K56 complemented with *KPHS_33520*, *KPHS_33590* and *KPHS_35510*, each designated K56::ZpUC19-*KPHS_33520*, K56::ZpUC19-*KPHS_33590* and K56::ZpUC19-*KPHS_35510*, showed increased virulence with less than 10% of the larvae remaining after 4 days (Figure 3.11). No significant difference was observed in other complemented strains. Therefore, it could be concluded that the positive candidate gene *KPHS_33600*, was virulent in *G. mellonella* larvae along with *KPHS_33520*, *KPHS_33590* and *KPHS_35510*.

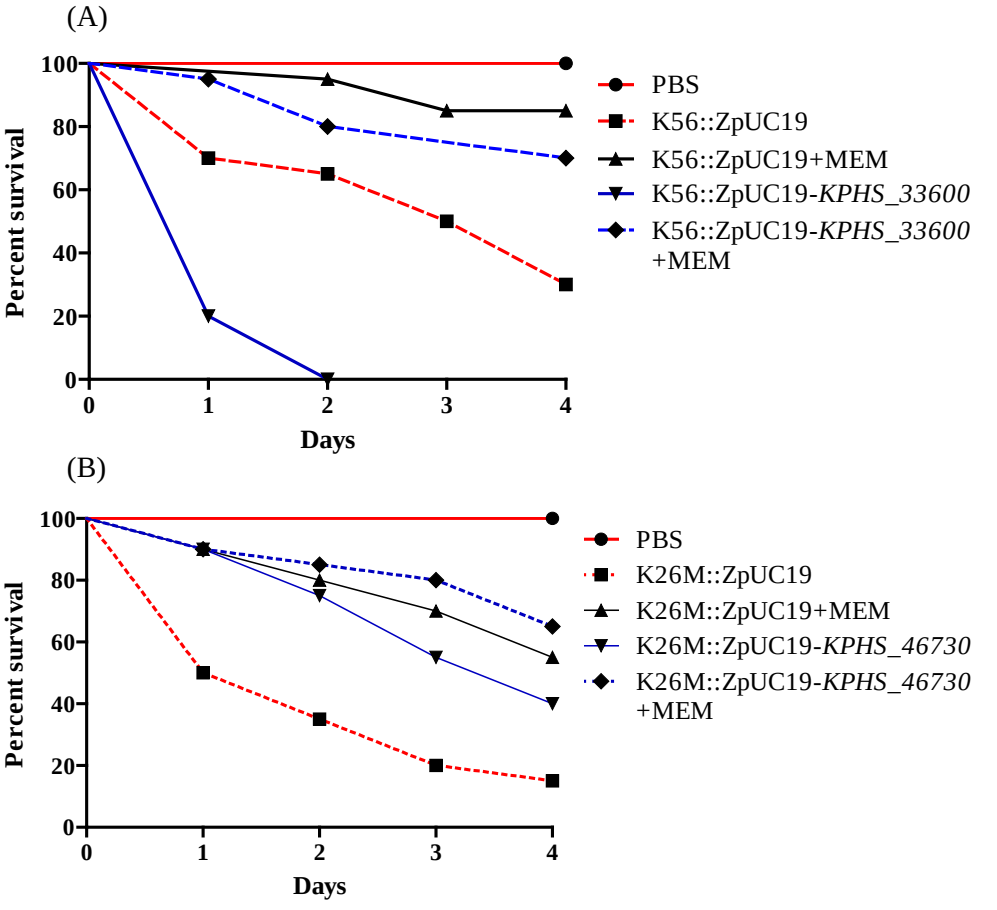

Figure 3.10. Virulence study for candidate genes in *G. mellonella* larvae. Larvae were infected with approximately 1×10^6 CFU of bacteria. After 4 hr of infection, meropenem was injected and the larvae were observed for four days. (A) Larvae infected with K56::ZpUC19-*KPHS_33600* (B) Larvae infected with K26M::ZpUC19-*KPHS_46730*.

Meropenem treatment was administered 4 hr after bacterial infection. Increased larvae survival was observed for both candidate genes (Figure 3.10(A), 3.10(B)). Interestingly, 70% increase in survival of larvae infected with K56::ZpUC19-*KPHS_33600* strain between treated and untreated could be observed. Similarly, 25% more larvae survived when K26M::ZpUC19-*KPHS_46730*-infected larvae were treated with meropenem. However, other three virulent strains did not show change in survival, regardless of whether meropenem was administered or not, with a small exception for K56::ZpUC19-*KPHS_33520*. In K56::ZpUC19-*KPHS_33520*, a few larvae recovered after the meropenem treatment, however, the difference was not significant compared to the untreated K56::ZpUC19 strain.

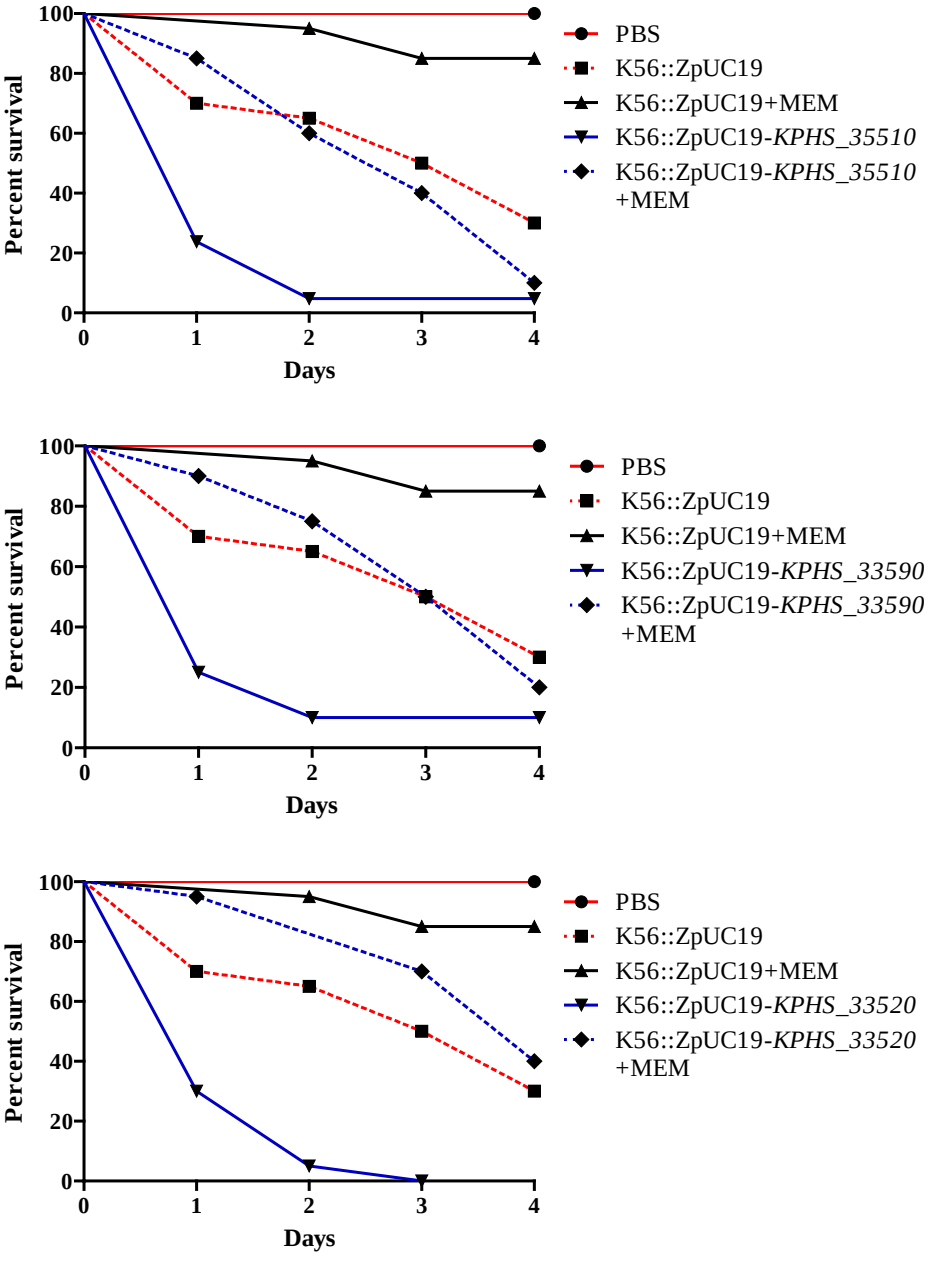

Figure 3.11. Genes responsible for virulence as observed in *G. mellonella* larvae. *KPHS_33590*, *KPHS_35510* and *KPHS_33520* complemented strains showed increased virulence in *G. mellonella* larvae compared to K56::ZpUC19. The larvae did not recover even after meropenem treatment for K56::ZpUC19-*KPHS_33590* and K56::ZpUC19-*KPHS_35510*.

IV. DISCUSSION

There is an urgent need to discover novel resistance mechanisms in order to overcome problems posed by the rise of antimicrobial resistant bacteria. In this study, novel uncharacterized efflux pumps were found to increase carbapenem susceptibility. Drug efflux in Gram-negative bacteria is accomplished by the collective workings of three components, i.e. an inner membrane transporter, a periplasmic MFP and an outer membrane channel.[52] *KPHS_33600*, a MFS transporter, was the candidate for in-vivo resistant strain K56 to partially bring about meropenem susceptibility. Down-regulation of the neighboring genes *KPHS_27260* and *KPHS_06070*, annotated as auxiliary transport protein-membrane fusion protein (MFP) family and HlyD family secretion protein respectively, indicated that they may be parts of an uncharacterized efflux pump which is down-regulated when complemented with *KPHS_33600*, thus decreasing the meropenem MIC. BLASTP search of *KPHS_06070* showed 95% sequence identity with HlyD family protein EmrA, which is an essential component of EmrAB-TolC MFS-dependent efflux pump in *E. coli*.[49] EmrA is a MFP which acts as a periplasmic adaptor and shares structural homology with AcrA, which plays an important role in β-lactam efflux in AcrAB-TolC RND type efflux transport system. Drug efflux takes place through TolC, the outer membrane channel, common for both EmrA and AcrA adaptors.[52] Therefore, we can hypothesize that complementation of *KPHS_33600* down-regulates the efflux pump required for meropenem efflux, thus making the *K. pneumoniae* strain partially meropenem-susceptible.

Outer membrane protein OmpK36 in *K. pneumoniae* plays an important role in carbapenem resistance.[53] OmpK36, when complemented into resistant strains did not change the meropenem MIC. This proves that OmpK36 porin loss does not affect meropenem susceptibility in carbapenemase non-producing carbapenem-resistant *K. pneumoniae* clinical isolates. Since all three strains had the same β-lactamase genes present in them, their involvement was ruled out. This finding opens door for further studies to find other gene combinations for carbapenem resistance.

KlebNet, a functional gene network, was constructed to further select the differentially expressed genes for complementation study based on their function. It covers 89% of the genes consisting of 5,316 coding genes with 213,516 co-functional links. This network links genes having the same or similar biological functions. Two candidates among 14 short-listed genes showed meropenem susceptibility, thereby demonstrated the accuracy of the network. Neighboring genes of *KPHS_33600* found using KlebNet provided insight into the gene function and hence the cause for meropenem susceptibility could be established. Therefore, KlebNet can be used to effectively find functional related genes of uncharacterized genes to connect them to known networks.

G. mellonella larvae, an insect model, is gaining popularity because of the several advantages it has over the traditional mammalian model. First and foremost, unlike mammalian models, larvae do not require approval from the ethics board. In addition, *K. pneumoniae* has been previously validated in this model[51] and the larvae can be incubated at 37℃ thus mimicking the condition of pathogenic infection in

humans.[54-57] In this study, virulence of the bacteria could be clearly observed in the larvae. Three genes namely, *KPHS_33510*, *KPHS_33590* and *KPHS_33520* showed increased virulence after complementation into K56. *KPHS_35510*, named *uge* (uridine diphosphate galacturonate 4-epimerase), is involved in colonization and virulence in mice model.[58] The sequence identity was 98.2% with BLASTP search. *KPHS_33590*, annotated as IclR family transcriptional regulator, has sequence identity of 87.1% with *KdgR* gene which is present in plant pathogen *Erwinia* sp.[59] However, there was no prior data on *KPHS_33520* (ribosomal RNA large subunit methyl transferase A) and its virulence. Further studies of this gene needs to be carried out to elucidate its mechanism of virulence.

A recent study showed discrepancy between results from studies using the *G. mellonella* larvae model and human patients infected by carbapenemase-producing *K. pneumoniae* model.[38] The larvae had survived infection with KPC producing strains while patients exhibited higher mortality from infection by the same strain. Almost 25% more KPC negative bacteria died compared to KPC-producing *K. pneumoniae*. They hypothesized that less fit organisms are more likely to acquire resistance and assume the associated fitness cost for plasmid carriage. Therefore, it can be concluded that the experimental results in-vivo may not completely replicate that within humans. When larvae were infected with K56::ZpUC19-*KPHS_33600*, the result was 100% larval death within 48 hr which recovered upon meropenem treatment. Therefore, virulence exhibited by the bacteria was very specific to the larvae. Further studies in

mice model needs to be carried out to validate the usefulness of *G. mellonella* larvae model for studying *K. pneumoniae* infections in humans.

One of the major limitation of this study is the inability to delete *KPHS_33600* and *KPHS_46730* genes from the susceptible strain K26. Clinical isolates are difficult to manipulate as required for research because they carry many resistant genes and mutations. K26 strain was resistant to all known antibiotic marker genes thus limiting our study. Therefore, mutants for these strains were obtained from Manoil Lab at University of Washington, USA. However, there was no difference between parent and the mutants (data not shown). This leads to the conclusion that either the candidate genes are strain-specific or they need additional gene deletion to express the meropenem-susceptible phenotype.

V. CONCLUSION

In conclusion, this study identified two novel genes that brought about increased meropenem susceptibility in carbapenemase non-producing carbapenem-resistant *K. pneumoniae* strains. *garL* complementation up-regulated the TCA cycle enzymes, thereby increasing the hydroxyl ions responsible for bacterial death. *KPHS_33600*, an uncharacterized MFS transporter, down-regulated EmrAB-TolC efflux pump, thus decreasing the meropenem MIC. However, the exact mechanism of action of *KPHS_33600* still needs to be further studied. Complete characterization is necessary to further understand the actual role of this gene.

CHAPTER IV

Limited performance of MALDI-TOF MS and SDS-PAGE for detection of outer membrane protein OmpK35 in carbapenem resistant *Klebsiella pneumoniae*

Limited performance of MALDI-TOF MS and SDS-PAGE for detection of outer membrane protein OmpK35 in carbapenem-resistant *Klebsiella pneumoniae*

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I. INTRODUCTION

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is used for rapid bacterial identification[60] in hospital settings, and some studies have validated its use in the detection of outer membrane proteins (OMPs) or porins.[61,62] Porins are a component of the outer membrane of gram-negative bacteria that play an important role in diffusion of bacterial nutrients and antimicrobials across the outer membrane.[63] *K. pneumoniae* is an opportunistic gram-negative pathogen and a common nosocomial microbe with a mortality rate of 50%.[17] It is worrisome to find increased numbers of carbapenem-resistant *K. pneumoniae* strains because carbapenems are considered to be the last-resort antibiotics. The resistance mechanisms of carbapenem-resistant *K. pneumoniae* have been attributed to loss of either one or both of the porins, OmpK35 and OmpK36,[64,65] in combination with the production of ESBLs or production of carbapenemase enzyme. Although OmpK35 in *K. pneumoniae* is homologous to OmpF in *Escherichia coli*, the porin channel is significantly larger than in *E. coli*.[66]

Therefore, lack of OmpK35 increases the multi-drug resistance in *K. pneumoniae*. At present, detection of porins using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is considered the gold standard and widely performed. Even though the process is laborious and time-consuming, the data obtained have been regarded as accurate. However, a recent study indicated discrepancies in results from *K. pneumoniae*; specifically, OmpK35 band was not detected in SDS-PAGE, while the corresponding peak was detected using MALDI-TOF MS.[62] It was therefore concluded that MALDI-TOF MS was a better detection method. However, the study was confined to the correlation between SDS-PAGE and MALDI-TOF MS without whole-genome sequence analysis or examination of protein expression levels. The aim of the present study was to validate the results obtained from SDS-PAGE and MALDI-TOF MS using whole-genome sequencing (WGS) and transcriptome analysis of carbapenem non-susceptible *K. pneumoniae* strains and to ascertain the reproducibility of MALDI-TOF MS. The performance of MALDI-TOF MS has been evaluated and compared using two different instruments, Microflex LT and Tinkerbell LT (ASTA, Suwon, Korea) mass spectrometers.

The writing and figures have been replicated from the published paper in *Oncotarget* in accordance with the license used by them.[67]

II. MATERIALS AND METHODS

1. Bacterial strains and identification

Eleven *K. pneumoniae* strains were used in this study including four carbapenem non-susceptible strains, one susceptible isolate ATCC 13883, and six other isolates that were a panel of strains from our laboratory consisting of both carbapenem non-susceptible and susceptible strains (Table 4.1). In this study the term 'carbapenem resistant' implies that the strain is resistant to at least one of the carbapenems, namely ertapenem, meropenem, or imipenem. The isolates were identified using a Bruker MALDI Biotyper CA System.

2. Antibiotic susceptibility testing

The MIC of carbapenems for the aforementioned eleven strains was determined using the agar dilution method and E-test, which were interpreted as described in the Clinical Laboratory Standards Institute (CLSI) guidelines.[68]

3. Outer membrane protein (OMP) extraction and analysis

OMP samples were extracted using both low-nutrient broth and high-osmolarity LB broth as previously described.[69] The obtained OMP sample was heated for 5 min at 100°C and placed on ice immediately. Extracted samples were separated using SDS-PAGE at a constant voltage of 60 V for about 3 hours in both a 12% gel and a gradient gel (ExpressPlus™ PAGE Gels, GenScript, Piscataway, NJ, USA). The bands were detected using Coomassie brilliant blue R-250 staining.

4. Detection using MALDI-TOF MS

The OMP samples extracted for SDS-PAGE were also used for detection by MALDI-TOF MS. The matrix was 40 mg/ml dihydroxybenzoic acid with 4.44 mg/ml 2-hydroxy-5-methoxybenzoic acid [9:1, w/w] in TA30 (30:70 [v/v] Acetonitrile: TFA 0.1% in water). The samples were analyzed using both Microflex LT and Tinkerbell LT mass spectrometry.

For Microflex LT, sample was diluted 10X before addition of the matrix. The diluted sample was then mixed with the matrix at a ratio of 1:1, and 1µl of the mixture was applied to the plate and air dried. The parameters for Microflex LT were as follows: mode, linear positive mode 10-50 kDa; ion source voltage 1, 20 kV; ion source voltage 2, 18 kV; lens voltage , 5 kV; linear detector voltage, 2.85 kV; pulsed ion extraction delay, 250 ns; digitizer trigger level, 5 mV; laser beam attenuation, 1.852; laser range, 70%; laser offset, 15%; sample rate, 2 ns; electronic gain, 100 mV; laser beam focus, -1; laser frequency, 60 Hz; number of shots, 500. Protein calibration standard I was used for calibration with a regulated calibration error of 67.5 ppm. The peaks were analyzed using flexAnalysis 3.4 (build 57) software.

For Tinkerbell LT, undiluted sample was mixed with the matrix at the same ratio, and 2 µl of the mixture was applied to the plate and air dried. The following conditions were maintained for Tinkerbell LT MALDI-TOF MS: range 15 kDa to 50 kDa, laser power 100%, shots 80×80, locus pattern, edge bias, radius 1,100 µm, delay time 3030, and smoothing of 13 points with baseline subtraction for peak processing. Extraction voltage of 18 kV and voltage gradient of 94.7% (17.05 kV) was

maintained. Bovine serum albumin protein standard was used for calibration i.e. 33 kDa and 22 kDa representing $[M+2H]^+$ and $[M+3H]^+$, respectively. The biological and technical repeats using Tinkerbell LT were done thrice.

5. Peptide analysis using LC-MS/MS and database searching

The putative OmpK35, OmpK36, and empty gel bands (at the height of band no.10) were excised from the SDS-PAGE gel for peptide analysis. The analysis was performed as previously described[70] by the Yonsei Proteome Research Center, South Korea. A nano-HPLC system (Agilent, Wilmington, DE, USA) was used for nano LC-MS/MS analysis. Peptide separation was carried out using a nano chip column. Product ion spectra were analyzed using Agilent 6530 Accurate-Mass Q-TOF.

The MASCOT algorithm (Matrix Science, UK) was used for database searching to identify protein sequences. The criteria used were, taxonomy; *Proteobacteria* (NCBInr downloaded 2015.01.23, OmpK35 (Accession no. ADG27468), OmpK36 (Accession no. YP_005228001) fixed modification: carbamidomethylated at cysteine residues; variable modification: oxidized at methionine residues; maximum allowed missed cleavage: 2; MS tolerance: 100 ppm; MS/MS tolerance: 0.1 Da.) Only peptides obtained from trypsin digestion were considered.

6. WGS and analysis

Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) was used for DNA extraction according to the manufacturer's protocol. The Qubit dsDNA BR assay kit (Molecular Probes, Eugene, OR, USA) was used to estimate DNA

concentration. Library preparation was carried out using an Ion Plus Fragment Library Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. WGS was carried out on a 318 chip v2 using the Ion Torrent PGM system and Ion Sequencing 400 kit (Life Technologies).

The obtained reads were assembled using MIRA plug-in. RAST annotation pipeline was used for annotation.[36] Genome analysis was carried out using Geneious 8.1.8 (http://www.geneious.com). Screening of β-lactamase genes in WGS was carried out using Resfinder (https://cge.cbs.dtu.dk/services/ResFinder/) and further verified using NCBI BLAST.

The protein molecular weights of all OMP peptides were calculated using protein calculator (https://spin.niddk.nih.gov/clore/Software/A205.html).[71]

7. RNA extraction, sequencing and data analysis

RNA was extracted using the RNeasy mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. DNA contamination was eliminated using an RNase-free DNase Kit. The concentration of RNA was measured using Nanodrop. RNA sequencing was performed using Illumina HiSeq2500, and transcriptome analysis were carried out using CLRNASeq software (http://www.chunlab.com/software_clrnaseq_download, Chunlab, Seoul, Korea). The data were normalized using Reads Per Kilobase per Million mapped reads (RPKM), Relative Log Expression (RLE), and trimmed mean of M-value (TMM) methods. *K.*

pneumoniae ATCC 13883 was used as the reference strain (Assembly ID: GCA_000742135.1).

8. Accession numbers

The GenBank accession numbers for *OmpK35* and *OmpK36* genes from YMC2014/1/R777, YMC2014/3/P345, YMC2014/4/B5656, and YMC2014/5/U865 are KY019185, KY019186, KY019187, KY019188 and KY019189, KY019192, KY019190, KY019191, respectively.

III. RESULTS

1. WGS and transcriptome analysis of the isolates

Data on MIC, β-lactamase genes and porins for all isolates are given in Table 4.1. Sequence analysis of all the four carbapenem non-susceptible isolates showed that OmpK35 was absent in all isolates due to deletion of a nucleotide at the 54th position leading to early termination of the gene. Sequence analysis also indicated that the OmpK36 porin in the strain YMC2014/03/P345 was interrupted by transposon insertion that hindered its expression, whereas no mutations were observed in the other three isolates. TMM was used to normalize the transcriptome values for this study because the coefficient of variation (CV) was 0.3387, which was lower than the values of 0.342 and 0.3396 for RPKM and RLE, respectively. The normalized data for *OmpA*, *OmpK35*, and *OmpK36* genes in carbapenem non-susceptible isolates using TMM are shown in Figure 4.1. The TMM values indicate that OmpK35 porin expression values were obtained, despite truncation, in all of the isolates, whereas in YMC2014/03/P345 the OmpK36 expression was found to be negligible.

Table 4.1. Characteristics of the strains used in this study

Strain	MIC (μg/mL)			β-Lactamase genes present in WGS	Band in MALDI TOF MS/SDS-PAGE				Mutation of porin genes in WGS			References
	IPM	ETP	MEM		36,200 m/z*/ 35.5 kDa*	37,500 m/z**/ 36 kDa**	38,200 m/z***/ 37 kDa***	38,400 m/z / 37.1 kDa	*OmpA*	*OmpK35*	*OmpK36*	
QC Strain												
ATCC 13883	0.5	≤0.5	≤0.25	bla_{SHV-1}	+/+	+/–	+/+	–/+	NM	NM	NM	This study
Carbapenem-resistant *K. pneumoniae*												
YMC2014/1/R777	1	**4**	0.5	bla_{SHV-12}, bla_{DHA-1}, bla_{LEN-11}	+/+	–/–	+/+	–/–	NM	54T deletion	NM	This study
YMC2014/3/P345	**>32**	**≥8**	**≥16**	bla_{SHV-12}, bla_{DHA-1}, bla_{LEN-11}	+/+	–/–	–/–	–/–	NM	54T deletion	Transposon insertion	This study
YMC2014/4/B5656	0.75	**4**	≤0.25	bla_{SHV-11}, bla_{DHA-1}	+/+	–/–	+/+	–/–	NM	54T deletion	NM	This study
YMC2014/5/U865	0.5	**4**	1	bla_{SHV-11}	+/+	–/–	+/+	–/–	NM	54T deletion	NM	This study
Panel strains of *K. pneumoniae*												
YMC2011/7/B36	1	0.75	0.25	bla_{SHV-11}, bla_{SHV-12}, bla_{DHA-1},	+/+	–/–	+/+	–/–	NM	54T deletion	NM	35
YMC2011/7/B774	0.25	1	0.25	bla_{OXA-1}, $bla_{CTX-M-15}$, bla_{SHV-11}, bla_{TEM-1}	+/+	–/–	–/–	–/–	NM	NM	Multiple point mutations	35
YMC2013/6/B3993	0.25	0.5	0.25	bla_{TEM-1}, bla_{SHV-12}, $bla_{CTX-M-15}$, bla_{OXA-9}	+/+	–/–	+/+	–/–	NM	*IS1* insertion	NM	74
YMC2011/8/B10311	0.5	**2**	2	bla_{TEM-1}, bla_{SHV-11}	+/+	–/–	–/–	–/–	NM	*OmpK35_v2* variant	Multiple point mutations	35
YMC2011/11/B1440	1	1	1	bla_{SHV-11}, bla_{SHV-12}, bla_{DHA-1}	+/+	–/–	+/+	–/–	NM	54T deletion	NM	35
YMC2011/11/B7578	1	**4**	1	bla_{SHV-12}, bla_{DHA-1}, $bla_{SHV-158}$	+/+	–/–	–/–	–/–	NM	54T deletion	313G deletion	35

WGS, whole-genome analysis; +, present; −, absent; NM, no mutation.

*, OmpA; **, OmpK35; ***, OmpK36

IPM, imipenem; ETP, ertapenem; MEM, meropenem

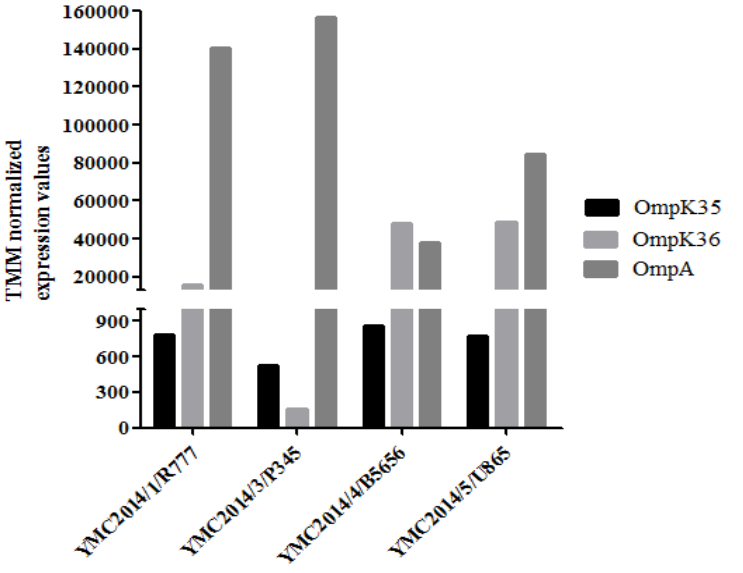

Figure 4.1. Trimmed mean of M-values of carbapenem-resistant strains. TMM-normalized values of the four carbapenem non-susceptible *Klebsiella pneumoniae* isolates for *OmpA*, *OmpK35*, and *OmpK36* gene expression.

2. OMP detection by using SDS-PAGE

For OMP analysis using SDS-PAGE, the samples were extracted from high-osmolarity LB broth and separated using 12% polyacrylamide and gradient gels. The separation was found to be similar in both types of gel (Figures 4.2a and 4.2b). We focused on the bands between 30 kDa and 38 kDa, where OmpK35 and OmpK36 are usually present. We found that for the YMC2014/03/P345 strain the OmpK36 band was absent in both gels, whereas the other three carbapenem non-susceptible isolates show two discrete bands. In detail, SDS-PAGE showed a prominent band at ~35.5 kDa in all four non-susceptible strains, which was previously considered to be OmpK35.[61,72] Surprisingly, this band was identified to be OmpA using LC-MS/MS.

Thus, all isolates lacked OmpK35 porin expression when analyzed using SDS-PAGE which is consistent with the WGS data. Extraction of OMPs was also carried out using low-osmolarity nutrient broth because a high expression of OmpK35 in low-osmolarity media was reported previously.[73] To confirm our findings, samples extracted from low-osmolarity nutrient broth were also run on a 12% polyacrylamide gel. It is interesting that the *K. pneumoniae* ATCC 13883 control strain showed an additional band (Figure 4.2c) above the OmpK36 band that was identified to be a combination of OmpK35 and OmpK36. This additional band migrating behind the OmpK36 band might reflect different migration patterns of porins between strains irrespective of molecular weight.[69]

Six random *K. pneumoniae* isolates (YMC2011/7/B36, YMC2011/7/B774, YMC2013/6/B3993, YMC2011/8/B10311, YMC2011/11/B1440, YMC2011/11/B7578) were selected from the panel strain bank (Table 4.1).[35,74] YMC2013/6/B3993 isolate lacked the *OmpK35* gene due to insertion of *IS*1 in the gene. The OmpK35 was also found to be truncated in YMC2011/7/B36, YMC2011/11/B1440, and YMC2011/11/B7578 due to the deletion of single nucleotide, similar the other carbapenem non-susceptible isolates. Only YMC2011/7/B774 and YMC2011/8/B10311 had intact OmpK35 in their WGS. But the band patterns for all of the panel strain isolates in SDS-PAGE did not show any signs of OmpK35. Only a single band representing OmpA was expressed in YMC2011/7/B774, YMC2011/8/B10311, and YMC2011/11/B7578 isolates because they lacked the OmpK36 porin (Table 4.1). Since no additional band was seen in

YMC2011/7/B774 and YMC2011/8/B10311, despite the absence of Coomassie blue staining, empty gels from these isolates were excised at the same height as that of band 10 and identified using LC-MS (data not shown). The proteins were identified to be a mixture of bisphosphate aldolase and OmpA while OmpK35 was completely absent in the excised gel.

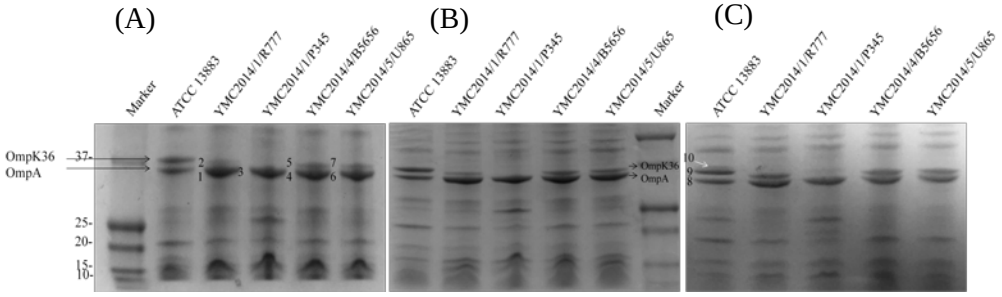

Figure 4.2. SDS-PAGE analysis of outer membrane proteins extracted from four carbapenem-resistant *K. pneumoniae* strains and the carbapenem-susceptible strain ATCC 13883 using various separation gels and growth media. (A), ExpressPlus™ PAGE 12% (w/v) polyacrylamide gel, isolates grown in Luria–Bertani broth; (B), ExpressPlus™ PAGE 4–12% gradient gel, grown in LB broth; (C), ExpressPlus™ PAGE 12% (w/v) gel, grown in nutrient broth. The bands numbered 1 to 10 represent the bands excised for liquid chromatography mass spectrometry. Bands 1, 3, 4, 6, and 8 represent OmpA; bands 2, 5, 7, and 9 represent OmpK36; band 10 contains both OmpK35 and OmpK36.

3. OMP detection using MALDI-TOF MS

For MALDI-TOF MS analysis of the OMPs, the samples extracted from high-osmolarity LB broth did not give reproducible results when analyzed by Microflex LT or Tinkerbell LT MALDI-TOF MS (data not shown). OMPs extracted from isolates grown in low-osmolarity nutrient broth were further analyzed using both Tinkerbell LT (Figure 4.3 and 4.4) and Microflex LT (Figure 4.5). The data obtained from both the instruments were consistent with each other. Four peaks at ~18 kDa, ~19 kDa, ~36 kDa, and ~38 kDa were observed in Tinkerbell LT (Figure 4.3). The peaks at ~18 kDa and ~19 kDa were considered to be multi-charged states of the ~36 kDa and ~38 kDa peaks, respectively. We presumed that the ~36 kDa peak represents OmpK35, while the ~38 kDa peak represents OmpK36, based on findings from a previous study.[61] But the four carbapenem non-susceptible isolates lacked OmpK35 and yet had a prominent ~36 kDa peak. By correlating to the peptide sequencing data, we could conclude that the ~36 kDa peak was in fact OmpA and not OmpK35. Consistent with WGS and SDS-PAGE findings, YMC2014/03/P345 lacked the peak at ~38 kDa, indicating the lack of OmpK36 porin expression. Three peaks were detected for *K. pneumoniae* ATCC 13883 (Figure 4.3), corresponding to bands no. 8, 9, and 10 as observed on the SDS-PAGE gel (Figure 4.2c). The additional ~37 kDa peak corresponding to band 10 represents the OmpK35 porin. The peak detection using Tinkerbell LT was repeated using the above strains. When OMPs of *K. pneumoniae* panel strains were analyzed by MALDI-TOF MS, a prominent ~36 kDa peak was observed for the strains YMC2013/6/B3993, YMC2011/7/B36,

YMC2011/11/B1440, and YMC2011/11/B7578 lacking OmpK35 (Figure 4.4), indicating that the ~36 kDa peak indeed represented OmpA. It is interesting that the peak corresponding to OmpK35 was not detected in MALDI-TOF MS for YMC2011/7/B774 and YMC2011/8/B10311 isolates, though the gene was intact. This led us to conclude that MALDI-TOF MS replicated the results obtained from SDS-PAGE and did not provide any additional data.

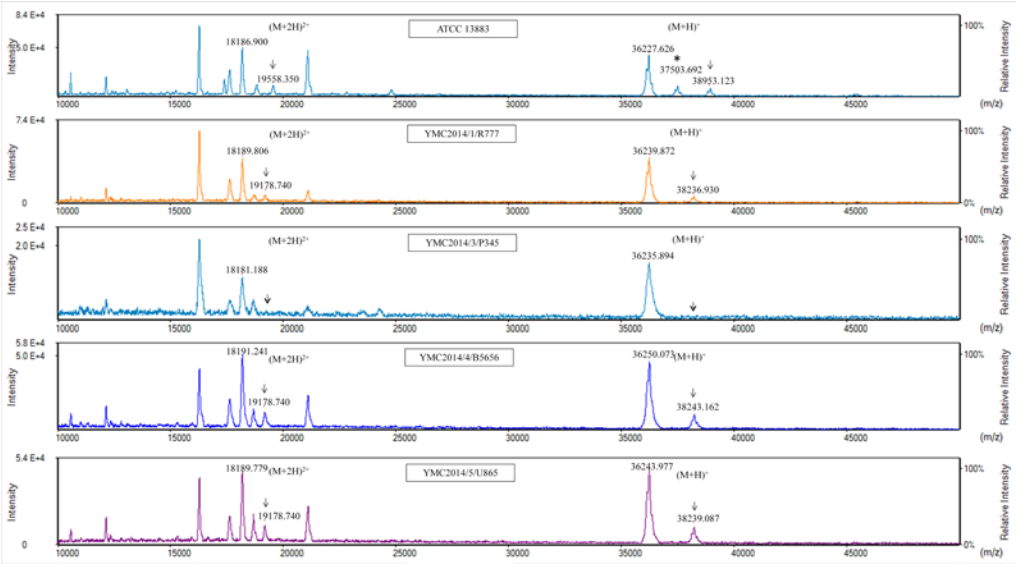

Figure 4.3. MALDI-TOF MS analysis of carbapenem-resistant *K. pneumoniae* isolates using Tinkerbell LT mass spectrometer. The x-axis represents the mass per charge in Daltons (m/z) and the y-axis represents the relative intensity. The 38 kDa peak and its corresponding $(M+2H)^{2+}$ peak at 19 kDa, indicated by solid black arrows, represent OmpK36. The dotted arrows indicate the loss of OmpK36. The peak at 36 kDa indicates OmpA. The asterisk (*) indicates the extra peak corresponding to sodium dodecyl sulfate polyacrylamide gel electrophoresis band 10 consisting of both OmpK35 and OmpK36.

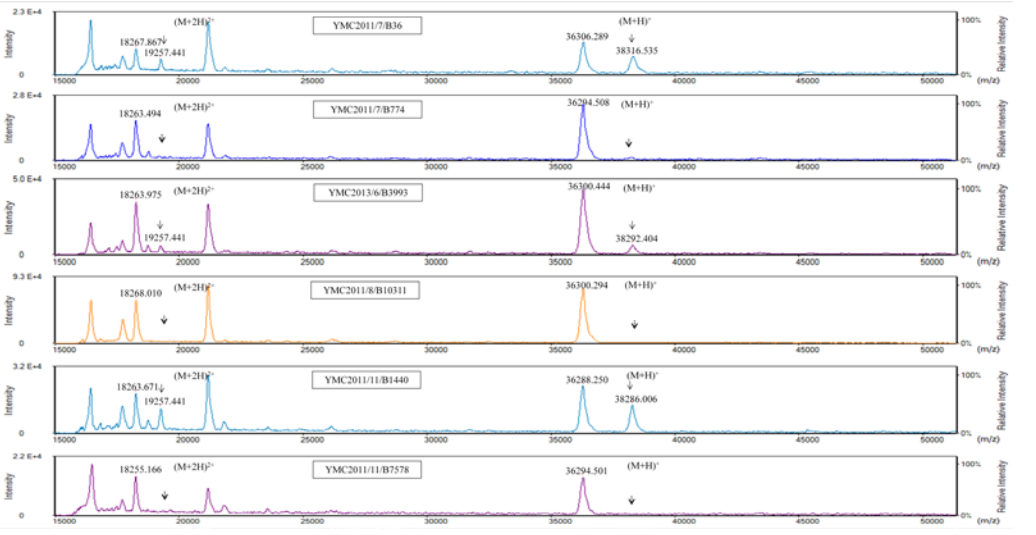

Figure 4.4. MALDI-TOF MS analysis of panel strains of *K. pneumoniae* using Tinkerbell LT mass spectrometer. The x-axis represents the mass per charge in Daltons (m/z) and the y-axis represents the relative intensity. The 38 kDa peak and its corresponding $(M+2H)^{2+}$ peak at 19 kDa, indicated by solid black arrows, represent OmpK36. The dotted arrows indicate the loss of OmpK36. The absence of the 37 kDa peak is indicated by the bold arrow in isolates YMC2011/7/B774 and YMC2011/8/B10311. Although these isolates carry the *OmpK35* gene, the corresponding peak for the OmpK35 protein was not observed.

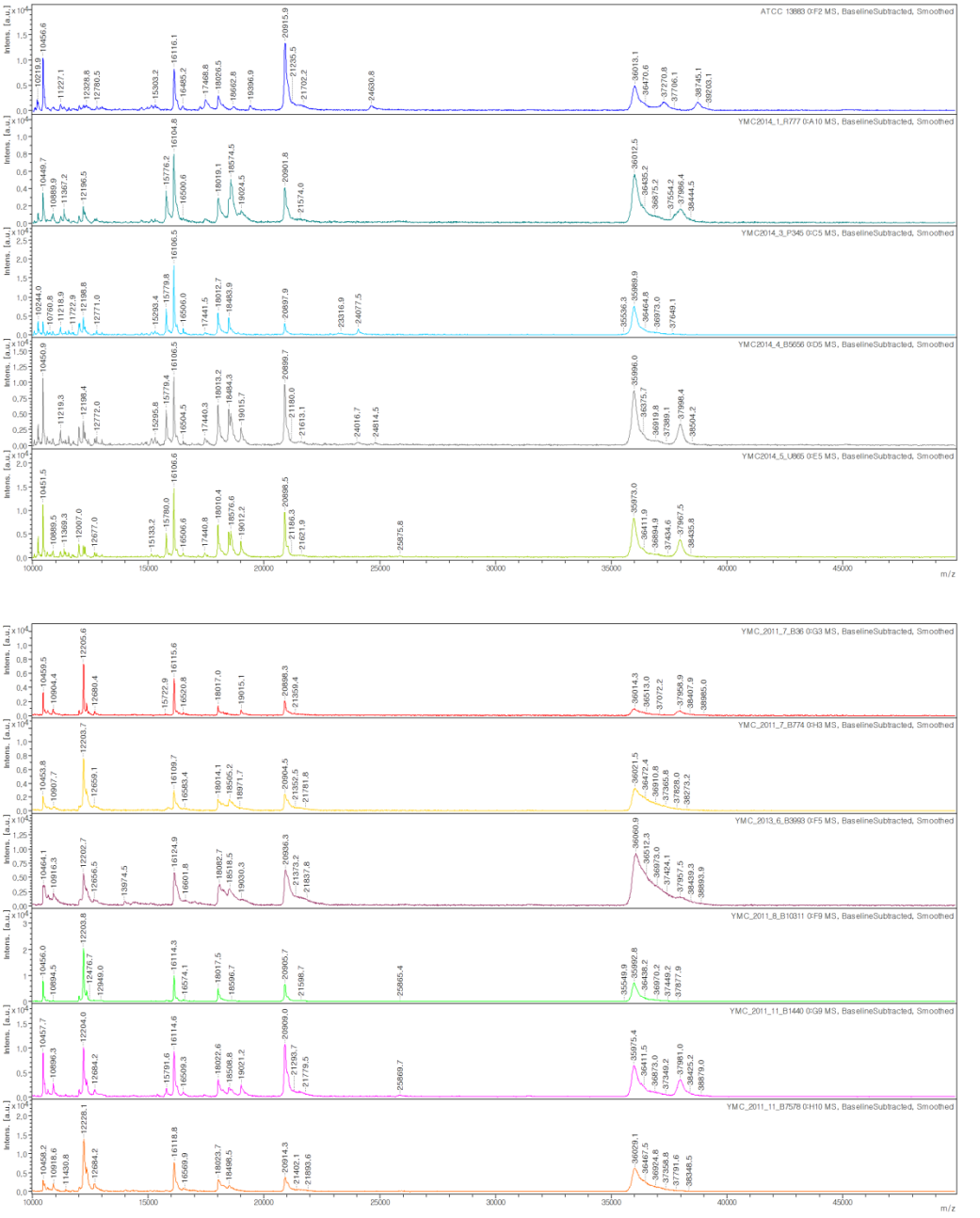

Figure 4.5. MALDI-TOF MS peaks obtained from analysis using a Microflex LT mass spectrometer. The peaks obtained are consistent with those obtained using a Tinkerbell LT mass spectrometer.

4. Protein molecular weight from WGS

The protein molecular weights of *OmpK35*, *OmpK36* and *OmpA* genes were calculated (Table 4.2). The expected molecular weights did not accurately correlate with the peaks obtained from MALDI-TOF MS. The calculated values were same with respect to OMPs in different isolates except for OmpK35 in YMC2011/8/B10311(variant named *OmpK35_v2*), due to the substitution of leucine to valine at position 14 in the leading peptide.[75] Since both leucine and valine belong to branched chain amino acid, the substitution did not affect OmpK35 expression.

Table 4.2. Predicted molecular weights of outer membrane protein gene products based on WGS data

Strain	OmpK35 (Da)	OmpK36 (Da)	OmpA (Da)
ATCC 13883	39509.46	40078.05	38975.79
YMC2014/1/R777	−	40078.05	38975.79
YMC2014/3/P345	−	−	38975.79
YMC2014/4/B5656	−	40078.05	38975.79
YMC2014/5/U865	−	40078.05	38975.79
YMC2011/7/B36	−	40078.05	38975.79
YMC2011/7/B774	39495.43	−	38975.79
YMC2013/6/B3993	−	40078.05	38975.79
YMC2011/8/B10311	39509.46	−	38975.79
YMC2011/11/B1440	−	40078.05	38975.79
YMC2011/11/B7578	−	−	38975.79

IV. DISCUSSION

MALDI-TOF MS is gaining momentum at present because of its rapid identification, cost-effectiveness and reliability. Recently, apart from bacterial identification, few studies have validated its use for the detection of OmpK35 and OmpK36 porins in *K. pneumoniae*.[61,62] However, our current study reveals the limitation of using MALDI-TOF MS in detection of OmpK35. We compared SDS-PAGE, WGS and transcriptome analysis results to provide sufficient data to validate our claims.

While whole genome analysis gives accurate genetic overview regarding mutations present in the porins, transcriptome analysis provides better insight into its expression. In this study, both SDS-PAGE and MALDI-TOF MS failed to detect OmpK35 in all clinical isolates, except *K. pneumoniae* ATCC 13883 strain. Therefore, our data is inconsistent with the previous study[62], where a peak at ~37 kDa in MALDI-TOF MS was always prominent even though the corresponding bands were absent in SDS-PAGE. In addition, absence of whole genome and transcriptome analysis of their data limits the scope for further comparison. The presence of OmpK35 band in *K. pneumoniae* ATCC 13883 may be because of the use of low-osmolarity media, i.e. nutrient broth, for enhancing the OmpK35 expression as reported previously.[73]

Although, WGS detected the presence of intact OmpK35 in YMC2011/7/B774 and YMC2011/8/B10311 isolates without truncation, no bands

were present in SDS-PAGE. LC-MS is more sensitive than Coomassie blue staining, and therefore, in order to identify whether OmpK35 peptides are present, bands were excised from the same height as that of band 10 (Figure 4.2c) in YMC2011/7/B774 and YMC2011/8/B10311 isolates. OmpK35 was absent irrespective of its isolation with nutrient broth. Instead, the bands were identified to be a mixture of bisphosphate aldolase and OmpA. In addition, no peaks were observed in MALDI-TOF MS for OmpK35 in these two isolates. This proves that MALDI-TOF MS reproduces the data obtained from SDS-PAGE.

The additional band in ATCC 13883, above OmpK36, which was a mixture of both OmpK35 and OmpK36 corresponds to the single 37 kDa band in MALDI-TOF MS. We believe that the band constitution and its location on the gel did not affect the position of the OmpK35 peak. Hu *et al*. (2015) described that peak observed at 37 kDa represented OmpK35 which was not expressed in SDS-PAGE. Their study had also analyzed *K. pneumoniae* ATCC 13883 and had identified a 37 kDa peak and its multi-charged state at 18.5 kDa. However, in our study, we were unable to find the multi-charged state of 37 kDa peak in *K. pneumoniae* ATCC 13883 and both OmpK35 peaks in YMC2011/7/B774 and YMC2011/8/B10311 isolates. Therefore, we spot a discrepancy in obtaining OmpK35 peak using MALDI-TOF MS.

In addition, the expected protein molecular weight of the OMPs did not correlate with the MALDI-TOF MS peak obtained (Table 4.2). This may be due to the direct analysis of extracted OMPs without further purification. The extracted OMP sample contains some amount of salts and other components from the buffer as

well as from the steps involved in the extraction procedure. This might suppress the signals from MALDI-TOF MS thus giving a difference in obtained peaks.[76]

One limitation of this study is that the transcriptome data available in our study was limited to carbapenem non-susceptible strains, which lacked OmpK35. The normalized values of OmpK35 might represent the expression of truncated proteins. This study also partially illustrates the limitation of using transcriptome data alone for data interpretation.

V. CONCLUSION

From the above analysis, two conclusions can be drawn: (i) MALDI-TOF MS replicates SDS-PAGE results for porin detection in *K. pneumoniae*. MALDI-TOF MS and SDS-PAGE show similar results even though they did not correlate with whole-genome and transcriptome data; for example, the failure to detect OmpK35. Thus to save time, MALDI-TOF MS can replace SDS-PAGE. Moreover, results obtained using Tinkerbell LT were replicable in low-osmolarity broth. (ii) Both MALDI-TOF MS and SDS-PAGE failed to detect peaks and bands representing the OmpK35 expression. In this regard, the data obtained in this study were inconsistent with previous studies. The inability of these methods to detect OmpK35 may be attributed to the detection limits of these two methods. This is the first report of the limitation of MALDI-TOF MS in detecting OmpK35.

Based on the above conclusions we stress that, although MALDI-TOF MS can replace SDS-PAGE for quicker analysis, neither of these methods can be used for porin detection in carbapenem non-susceptible or resistant *K. pneumoniae*. Changes to the OMP extraction process may yield better extracts that can be detected using SDS-PAGE or MALDI-TOF MS. Furthermore, studies that have previously identified OmpA band as OmpK35 without further confirmation should be re-evaluated.

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ABSTRACT (in Korean)

인체내와 시험관내에서 carbapenem 내성을 획득한 폐렴막대균의 관련유전자 규명

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항균제 내성은 세계적으로 보건 의료에 위기를 초래하고 있다.2050 년까지 항균제 내성 세균에 의한 사망자는 1000 만 명/ 년에 이르게 될 것으로 예상한다. 세균이 내성을 획득하는 속도는 새로운 항균제를 찾는 것을 능가하고 있다. 기회 감염균인 *Klebsiella pneumoniae* 는 많은 항균제에 내성이어서 치료가 어렵다. 따라서, 우리는 *K. pneumoniae* 를 통제할 수 있는 새로운 약물표적과 작용기전을 찾고자 하였다. 본 연구에서는 meropenem 으로 치료한 환자에게서 분리된, carbapenemase 를 생성하지 않으면서 carbapenem 에 내성을 보이는 *K. pneumoniae* 의 meropenem 항균제 내성 기전을 규명하고, porin 검출법을 평가하였다.

제 1 장에서는 항균제의 역사, 표적과 내성기전, β-lactam 계열 항균제, *K. pneumoniae*의 carbapenem 내성기전에 대하여 정리하였다.

제 2 장에서는 carbapenemase 비생성 carbapenem 내성 *K. pneumoniae* 임상 균주에서 3 차원 bioassay 검사에서 양성을 보인 원인을 설명하였다. 방사선으로 돌연변이를 유발하여 내성균주를 감수성으로 전환시킨 후, 3 차원 bioassay 검사에서 음성이 된 이유는 bla_{CMY-10} AmpC β-lactamase 유전자 소실 때문이었다. 이때 OmpK35 와 OmpK36 두 가지의 OMP 가 동시에 소실되고, bla_{CMY-10} AmpC β-lactamase 유전자를 보유하는 경우 meropenem 내성을 회복하였다.

제 3 장에서는 인체내 및 시험관내 carbapenem 내성 *K. pneumoniae* 가 meropenem 감수성을 갖게 되는 새로운 기전을 밝혔다. 이와 연관될 것으로 추정되는 유전자 목록을 전체 게놈 분석, transcriptome 분석, 기능 유전자 네트워크 (KlebNet)를 통하여 작성하였다. 인체내 내성유도균주 K56 와 시험관내 내성유도균주 K26M 에서 *KPHS_33600* (MFS transporter) 유전자와 *KPHS_46730* (*garL*) 유전자를 주입 (complementation) 하여 meropenem 최소억제농도(MIC)가 ≥32 μg/ml 에서 8 μg/ml 으로 감소하였다. 유전자를 주입하여 LB 배지에서 시행한 적합성

시험(fitness assay)에서 감소를 보이지 않았으며, *Galleria mellonella* 유충을 *KPHS_33600* 와 *garL* 주입균주로 감염시키고 meropenem 을 투여하여 성공적으로 치료하였다. *KPHS_33600* 와 *garL* 주입 균주로부터 얻어진 전사체 데이터를 이용하여 가능한 작용기전을 설명하였다.

제 4 장에서는 외막단백질(outer membrane protein, OMP) 검출을 위하여 MALDI-TOF MS, SDS-PAGE, 전유전체 분석 및 전사체(transcriptome) 결과를 비교하였다. 현재 외막단백질을 검출하기 위하여 SDS-PAGE 를 표준으로 사용하고 있다. OmpK35 는 SDS-PAGE 및 MALDI-TOF MS 를 사용하여 검출할 수 없었다. 그러나 두 방법에서 얻은 결과는 동일하여서, MALDI-TOF MS 가 SDS-PAGE 와 동등하였다. RNA 분석은 돌연변이 유전자의 발현이 낮았기 때문에 확인할 수 없었다. 전유전체 분석과 PCR 에 이은 Sanger sequencing 이 외막단백질을 정확하게 검출할 수 있었으므로, *K. pneumoniae* 균주에서 외막단백질을 검출하는 가장 신뢰할 만한 방법이었다.

결론적으로, 본 연구는 carbapenem 내성 *K. pneumoniae* 에서 항균제 내성기전과 외막단백질 분석을 위한 신뢰할 검출법을 찾으려 하였다.*Pseudomonas aeruginosa* PAO-1 와 *Escherichia coli* 와는 달리 *K.*

pneumoniae 는 연구된 바가 적다. 따라서, 아직 연구 관심의 대상이 되지 않은 여러 유전자가 있다. 특징 지어지지 않은 수송체 유전자 중에 하나인 *KPHS_33600* 과 meropenem 감수성과의 연관에 대한 추후 연구가 진행되어야 한다.

핵심되는 말: **폐렴막대균, *bla*$_{CMY-10}$, 방사선유발 돌연변이, 전장유전체 분석, KlebNet, 전사체 분석, 주외막단백질**

PUBLICATION LIST

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