Investigations on efflux pump and porin related genes in *Pseudomonas aeruginosa* selected from clinical isolates of multidrug resistant gram-negative bacteria

Thesis submitted to University of Calicut
in partial fulfillment of the requirement for the award of

Doctor of Philosophy
In
Biotechnology

By

MANJU SURESH

DEPARTMENT OF BIOTECHNOLOGY
UNIVERSITY OF CALICUT
2018

DEPARTMENT OF BIOTECHNOLOGY
UNIVERSITY OF CALICUT

Certificate

This is to certify that the Thesis entitled **"Investigations on efflux pump and porin related genes in *Pseudomonas aeruginosa* selected from clinical isolates of multidrug resistant gram-negative bacteria"** submitted to University of Calicut, as partial fulfillment of Ph.D. programme for the award of the degree of Doctor of Philosophy in Biotechnology by **Manju Suresh.,** embodies the results of bonafide research work carried out by her under my guidance and supervision at the Department of Biotechnology, University of Calicut. This Thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles or recognition. The candidate has passed the course work of the Ph.D. programme in accordance with the UGC regulations.

C.U Campus
Date:

Dr. P. R. Manish Kumar
(Research Supervisor)
Professor, Head and Coordiantor
Department of Biotechnology
University of Calicut
Kerala - 673635

DECLARATION

I hereby declare that the work presented in the Thesis entitled **'Investigations on efflux pump and porin related genes in *Pseudomonas aeruginosa* selected from clinical isolates of multidrug resistant gram-negative bacteria'** submitted to the University of Calicut, as partial fulfillment of Ph.D. programme for the award of the degree of Doctor of Philosophy in Biotechnology, is original and carried out by me under the supervision of Dr. P. R. Manish Kumar, Department of Biotechnology, University of Calicut. This has not been submitted earlier either in part or full for any degree or diploma of any university.

C.U Campus **Manju Suresh**
Date :

ACKNOWLEDGEMENTS

First and foremost, all my praises and thanks go to The Almighty for giving me the great opportunity and strength to successfully complete my Ph.D and for sustaining my efforts which many a times did oscillate.

It is a genuine pleasure to express my deep sense of thanks and gratitude to my mentor, philosopher and guide Dr. P.R. Manish Kumar. His untiring dedication, prompt inspirations, timely advise, meticulous scrutiny along with unwavering enthusiastic and dynamic scientific approach helped me to proudly accomplish my research.

My heartfelt love, respect and gratitude to my dear teacher Jayasree madam whose unconditional support from my college days till now made me worth achieving this task of my life.

I would like to extend my thanks to Prof. K K Elyas, Dr. Smitha Bava and Mr. Gopinathan, faculty of the department for their support.

My wholehearted thanks to my cousin brother Ajiettan, for intimating me about applying for INSPIRE fellowship which paved my way to research.

I am grateful to the Department of Science and Technology, Government of India, for awarding me INSPIRE fellowship that took care of all financial necessities during the tenure of my work.

I would like to express my sincere thanks to my senior, Dr. Rajan I. for his guidance in basic techniques, all out help and support during critical situations of troubleshooting - like an elder brother.

I also express my thanks to my senior Dr. Nithya N for her valuable suggestions and also providing me with some of the bacterial samples used in my study.

I would like to thank, with extreme happiness and love, my friend Shaniba who motivated me like a sister in every hurdle I faced during my tenure of research.

I am very happy to express my gratitude and affection to my sweet younger sister Ahlam and her husband Saifudheen. They have been very cooperative and

resourceful in helping me to overcome unforeseen obstacles in my research and life. I am forever indebted to them for prayers, compassion, sacrifices and love.

I would like to express my sincere gratitude to Soumya T, my labmate for her valuable help and support during my study.

I would also like to take this opportunity to thank my juniors, Jobish for his technical help and Meghna for her loving presence. I am also thanking all research scholars, PG students, Office and non-teaching staff members of the department for helping me in one way or other during my years of research.

I also acknowledge, our librarian, Mr. Iqbal for his help in various situations.

It would be my pleasure to express my heartfelt gratitude to my teacher Rajadheesh Sir and my friends Nijesh, Shabina and Ajith for the collection of bacterial isolates used in my study.

I am greatly indebted to Dr. Vimal, Department of Botany, University of Calicut for technical help PCR and with the phylogenetic analyses. I would also like to express my pleasure to thank Mr. Akhilesh, Department of Zoology, University of Calicut for his immense help with PCR.

I would like to convey my gratefulness to Associate Professor Dr. Sinosh Skariyachan (Department of Biotechnology, Dayananda Sagar College of Engineering, Bengaluru) for very generously extending his expertise in molecular modelling to me for my research work.

I would like to extend my immense gratitude to Dr. Anil (Dept. of Botany), Dr. Sreejith (Curator, Dept.of Zoology), Ms.Sreena Vimal (Dept. of Life Sciences) for their unconditional help in DNA sequence depositions.

Thanks to Faisal, Maya, Hareesh (Dept. of Botany) for helping me in my PCR work.

I also thank Mr. Rajesh, Bina Photostat for all technical assistance in preparing my Thesis.

This journey would not have been possible without the warmth, care and love of those who surrounded me. The blessings, prayers and support of all my family members encouraged me in all of my pursuits and inspired me to follow my dreams.

Also, I am deeply thankful to Sr. Floriana, my teacher whose blessings ignited my life and Gracy Aunty and her family for providing me with soothing and memorable moments in between the course of my work.

I am immensely obliged to my in-laws for their whole-hearted support to complete my goal.

Last but not least, I have no words to thank my better half, Mr. Krishnadas and my li'l toddler, Thejaswi for their patience, support, care, love and emotional relief given to me with which enabled me come through with flying colours.

Manju Suresh

Dedicated to

All My Teachers

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ABC	:	ATP-binding cassette
AHSLs	:	Acylhomoserine lactones
AIC	:	Akaike Information Criterion
AK	:	Amikacin
ALA/A	:	Alanine
AMP	:	Ampicillin
ANOLEA	:	Atomic Non-Local Environment Assessment
ARG/R	:	Arginine
ASN/N	:	Asparagine
AT	:	Aztreonam
ATP	:	Adenosine triphosphate
BLASTN	:	Basic Logical Alignment Search Tool for nucleotides
BLAST	:	Basic Logical Alignment Search
bp	:	Base Pair
b/w	:	Between
C	:	Chloramphenicol
CAC	:	Ceftazidime in combination with clavulanic acid
CAZ	:	Ceftazidime
CDDEP	:	Center for Disease Dynamics, Economics and Policy
cDNA	:	complementary DNA
CFU	:	Colony-forming unit
CIA	:	Chloroform/Isoamyl alcohol
CIP	:	Ciprofloxacin
CL	:	Colistin
CLSI	:	Clinical and Laboratory Standards Institutes
CPM	:	Cefepime
CT		Cycle threshold
CTX	:	Cefotaxime
CTAB	:	Cetyl Trimethyl Ammonium Bromide
D	:	Aspartic Acid
DHFR	:	Dihydrofolate reductase
DHPS	:	Dihydropteroate synthase
DMT	:	Drug/metabolite transporter
DNA	:	Deoxyribonucleic acid
DNase	:	Deoxyribonuclease
dNTP	:	Deoxynucleoside triphosphate

DTT	:	Dithiothreitol
E value	:	Expectation value
EDTA	:	Ethylenediaminetetraacetic acid
EF	:	Elongation factor
erm	:	Erythromycin ribosomal methylation
EPI	:	Efflux pump inhibitor
ESBL	:	Extended spectrum beta-lactamase
ESS	:	Estimated sample size
EtBr	:	Ethidium bromide
ExPASy	:	Expert Protein Analysis System
F	:	Phenylalanine
GEN	:	Gentamicin
GTE	:	Glucose Tris-EDTA
g	:	Gravity
GLASS	:	Global Antimicrobial Surveillance System
GLN/Q	:	Glutamine
GLU/E	:	Glutamic acid
GLY/G	:	Glycine
GROMOS	:	Groningen molecular simulation
h	:	Hour(s)
I	:	Isoleucine
ICMR	:	Indian Council of Medical Research
IIMAR	:	Indian Initiative for Management of Antibiotic Resistance
INSAR	:	Indian Network for Surveillance of Antimicrobial Resistance
IMTECH	:	Institute of Microbial Technology
INSAR	:	Indian Network for Surveillance of Antimicrobial Resistance
IPM	:	Imipenem
IS	:	Insertion sequences
Kdo	:	3-deoxy-D-manno-oct-2-ulosonic acid
KPC	:	*Klebsiella pneumoniae* carbapenemase
L	:	Loop region
l	:	litre
LB	:	Luria Bertani
LEU/L	:	Leucine
LPS	:	Lipopolysaccharides
LYS/K	:	Lysine
M (in gel Photographs)	:	Molecular Weight marker
MAR	:	Multiple Antimicrobial Resistance

MATE	:	Multidrug and toxic compound extrusion
MBL	:	Metallo beta-lactamase
MDR	:	Multidrug resistant
MEGA	:	Molecular Evolutionary Genetics Analysis
MET/ M	:	Methionine
MFP	:	Membrane fusion protein
MFS	:	Major facilitator superfamily
mg	:	Milligram
MHA	:	Mueller-Hinton agar
min	:	Minutes
ml	:	Milliliter
mM	:	Millimolar
MR	:	Methyl red
MRP	:	Meropenem
MRSA	:	Methicillin-Resistant *Staphylococcus aureus*
mRNA	:	messenger RNA
MTCC	:	Microbial Type Culture Collection
N	:	Normality
NA	:	Nalidixic acid
NaCl	:	Sodium Chloride
NEB	:	New England Biolabs
NAD^+	:	Nicotinamide adenine dinucleotide
NBDs	:	Nucleotide-binding domains
NCBI	:	National Center for Biotechnology Information
NDM-1	:	New Delhi metallo beta-lactamase-1
ng	:	Nanogram
NGOs	:	Non-governmental organizations
nm	:	Nano meter
OD	:	Optical density
OF	:	Ofloxacin
OM	:	Outer membrane
OMP	:	Outer membrane protein
ORF	:	Open Reading Frame
OXA	:	Oxacillinase
PACE	:	Proteobacterial antimicrobial compound efflux
PAN	:	Phenylalanine-arginine beta-naphthylamide
PB	:	Polymyxin B
PCR	:	Polymerase chain reaction
PDB	:	Protein data bank

PBP	:	Penicillin binding protein
PIT	:	Piperacillin/tazobactam
PROCHECK	:	Program to check the stereochemicai quality of protein structures
P	:	Proline
PSRF	:	Potential scale reduction factor
PyElph	:	A software tool for gel images analysis/phylogenetics which uses Python programming language
PyMOL	:	A molecular visualization computer software which uses Python programming language
QMEAN	:	Qualitative Model Energy Analysis
QRDR	:	Quinolone resistance determining region
RAPD	:	Random Amplified Polymorphic DNA
RFLP	:	Restriction fragment length polymorphism
RNA	:	Ribonucleic acid
RND	:	Resistance-nodulation-cell division
rpm	:	Revolutions per minute
RT-PCR	:	Reverse Transcription-PCR
s	:	Seconds
SCC*mec*	:	Staphylococcal cassette chromosome *mec*
SDS	:	Sodium dodecyl sulfate
SER/S	:	Serine
SMR	:	Small multidrug resistance
SMX	:	Sulfamethoxazole
spp	:	Species
SPSS	:	Statistical Package for the Social Sciences
STE	:	Sodium Chloride Tris-EDTA
TB	:	Tuberculosis
TBE	:	Tris-Borate-EDTA
TE	:	Tetracycline
TE buffer	:	Tris-EDTA buffer
THR/T	:	Threonine
TMDs	:	Transmembrane domains
TMP	:	Trimethoprim
TMS	:	Transmembrane-spanning segments
UDP	:	Uridine diphosphate
UPGMA	:	Unweighted pair group method using arithmetic averages
UV	:	Ultra-violet
V	:	Valine

VISA	:	Vancomycin-intermediate *S. aureus*
VP	:	Voges-Proskauer
VRSA	:	Vancomycin resistant *S. aureus*
v/v	:	volume/volume
W	:	Tryptophan
w/v	:	weight/volume
WHO	:	World Health Organization
Y	:	Tyrosine
3D	:	3 dimensional
3-oxo-C12-HSL	:	*N*-3-oxo-dodecanoyl-L-homoserine lactone
µg	:	Microgram
µl	:	Microliter

INTRODUCTION

Antibiotics - the 'miracle drugs' of the 20th century - continue to play a major role in curing infectious diseases. The discovery of 'Salvarsan', an arsenic based drug for syphilis in 1909, development of natural antibiotic penicillin in 1928 and synthesis of a sulpha drug, Prontosil in 1935, opened a golden era of novel antibiotic inventions. Alexander Fleming - the discoverer of the first natural antibiotic, penicillin - was also among the first who warned about the potential resistance to penicillin if used too little or for too short a period during treatment (Aminov, 2010). The ability of microbes to grow or survive in the presence of a concentration of antibiotic that is usually sufficient to kill them or inhibit their growth is termed as antibiotic resistance. Although antibiotic resistance can be considered to be a natural phenomenon driven by the selection pressure of antimicrobial agent, indiscriminate use of antibiotics is a major factor facilitating the emergence of resistance worldwide (Sabtu et al., 2015). Emergence of multidrug resistance, which is defined as non-susceptibility of bacteria towards at least one agent in three or more antimicrobial categories, has become a significant public health threat (Allcock et al., 2017).

1.1 Molecular mechanisms of antibiotic resistance

Bacteria can attain antibiotic resistance through intrinsic or acquired mechanisms. Intrinsic mechanisms are those specified by naturally occurring genes found on the host's chromosome and such resistance can be a result of impaired permeability of the bacterial envelope, efficient drug export systems, the absence or presence of low affinity antimicrobial target or the presence of enzymes which inhibit or destroy the antibiotics (Galán et al., 2013). Acquired resistance, can either be a consequence of *de novo* mutations or may arise due to acquisition of resistance determinants borne on plasmids, bacteriophages, transposons, and other mobile genetic elements by horizontal transfer. Acquisition of new genetic material is accomplished through the processes of conjugation, transformation, or transduction (Alekshun and Levy, 2007).

Mutations resulting in antibiotic resistance alter the antibiotic action via one of the following mechanisms, such as (i) antibiotic inactivation, (ii) changes in target site, (iii) active efflux and (iv) down regulation of outer membrane protein channel are all transferrable to subsequent generations - a phenomenon known as vertical evolution (Munita and Arias, 2016).

1.2 Role of porins and efflux pumps in multidrug resistant (MDR) gram-negative bacteria

Antibiotic resistance in gram-negative bacteria is attributable to a synergetic interplay between reduced drug intake due to low permeability of outer membrane and an active drug export consequent to upregulation of efflux pumps. These two aspects reportedly play a critical role in the development of the MDR phenotype (Fernández and Hancock, 2012).

Gram-negative bacteria are intrinsically resistant to many antibiotics due to the permeability barrier provided by their multifaceted cell envelope comprising of an outer membrane embedded with channels of both specific and non-specific types. Porins are nonspecific, water-filled open channels which act as molecular filters for hydrophilic substances, mediate transport of nutrients and ions including antibiotics across the membrane into the periplasm (Fernández and Hancock, 2012; Galdiero et al., 2012).

Bacterial efflux pumps are transporting systems lodged in cell membrane which drive out various compounds such as physiological substrates, non-antibiotic substrates as well as antibiotics from the cell. Multidrug efflux pumps are encoded by bacterial genomes and commonly belong to various families on the basis of their sequence similarity, substrate specificity, number of components (single or multiple), number of transmembrane-spanning regions and energy source. Currently, these have been categorized into six families: (i) the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily, (ii) the major facilitator superfamily (MFS), (iii) the small multidrug resistance (SMR) family, (iv) the multidrug and toxic compound extrusion (MATE) family (v) the resistance-nodulation-cell division (RND) superfamily and (vi) proteobacterial antimicrobial

compound efflux (PACE) family (Masi et al., 2017). The RND efflux pumps play a key role in the development of both intrinsic and acquired multidrug resistance in gram-negative bacteria (Blair and Piddock, 2009; Vargiu et al., 2016; Alibert et al., 2017). These pumps are comprised of a characteristic tripartite complex formed of a cytoplasmic membrane transporter, a membrane fusion protein (MFP) and an outer membrane protein (OMP) channel (Symmons et al., 2009; Daury et al., 2016; Puzari and Chetia, 2017).

1.3 *Pseudomonas aeruginosa*

P. aeruginosa, a 'priority pathogen' has now been included in a list of 12 families of bacteria which pose a serious health threat to man (WHO, 2017a). This opportunistic microbe is also held responsible for nosocomial infections worldwide. Known to possess a large number of virulence factors, it causes severe infections with high morbidity and mortality rate, particularly in immune-compromised patients or those with underlying disease (Poole, 2001; Strateva and Yordanov, 2009; Askoura et al., 2011; Porras-Gómez et al., 2012; Chatterjee et al., 2016). *P. aeruginosa* has now achieved a superbug status by acquiring multidrug-resistant phenotypes through (i) intrinsic resistance mechanisms such as those mediated by constitutive expression of AmpC beta-lactamase, efflux pumps and porin down-regulation and (ii) acquired resistance caused by mutational changes or acquisition of resistance mechanisms via horizontal gene transfer. Together, these processes contribute toward development of overwhelming resistance against a variety of structurally unrelated antibiotics leading to difficulties or failure in therapy (Poole, 2011). Efflux pumps of clinical relevance in *P. aeruginosa* belong to the RND family, of which MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY represent the predominant sets of efflux systems, with a broad range of drug specificities. *P. aeruginosa* infections are commonly treated with carbapenems which belong to the beta-lactam class of antibiotics. Carbapenem-uptake mainly occurs through OprD porin proteins. However, the occurrence of carbapenem-resistant strains is gradually increasing and recent studies indicate involvement of OprD down-regulation in combination with overexpression of efflux systems in the

development of resistance (Lee and Ko, 2012; Ocampo-Sosa et al., 2012; Fang et al., 2014; Zeng et al., 2014; Kim et al., 2016).

1.4 Selection pressure and dissemination of antibiotic resistance as a global phenomenon

Under the selective pressure of antibiotics, bacteria which are either resistant intrinsically or have acquired antibiotic resistant determinants possess a greater chance to survive and multiply. Overuse, inappropriate choices, inadequate dosing and poor adherence to treatment guidelines of antibiotics are the underlying causes of the widespread increased antibiotic resistance observed at a global level (Prestinaci et al., 2015). Antibiotics used in live stock and aquaculture for growth promotion, disease treatment and prophylaxis are considered to be the major contributors to the overall problem of resistance (Marshall and Levy, 2011). The majority of consumed antibiotics released into the environment through waste streams and wastewater treatment plants, are thought to be the evolutionary hotspots for antimicrobial resistance dissemination, since resistance genes, mobile genetic elements and antibiotic selection pressure from various sources are introduced to commensals and pathogens (von Wintersdorff et al., 2016).

1.5 Indian scenario of antibiotic resistance

Antibiotic resistance is a stark reality across the world including the Indian subcontinent. In the country the challenges associated with controlling antibiotic resistance are various and multifaceted. Disease burden in India is among the highest in the world due to the inappropriate and irrational use of antimicrobial agents and reports in 2010 showed India as the world's largest consumer of antibiotics for human health. Multiple factors, such as high disease burden, poor public health infrastructure, rising incomes, unregulated sales of cheap antibiotics and poverty among the low-income strata of society result in large-scale selection and dissemination of resistance genes in India (Laxminarayan and Chaudhury, 2016). Health sector in India suffers from gross inadequacy of public finance leading to favourable conditions for development of drug resistance. Also, systematic nationwide surveillance programme of antibiotic resistant pathogens in

various sectors is inadequate or lacking in India. Paucity of national repository of resistant pathogens makes it difficult to understand the severity of the problem and the involvement of various factors responsible for emergence of antimicrobial resistance (Kumar et al., 2013). Several new initiatives have been launched by various agencies to address this problem including, i) IndiaClen - Indian Clinical Epidemiology Network, which has generated data on resistance in pathogens like *Pneumococcus, Haemophilus influenzae* across the country, ii) IIMAR - Indian Initiative for Management of Antibiotic Resistance launched in March 2008, with WHO support, by a consortium of NGOs to promote prudent use of antimicrobials, iii) INSAR - Indian Network for Surveillance of Antimicrobial Resistance, a network of 20 laboratories in the private as well as public sectors across the country to generate quality data on drug resistance, iv) organization by the ICMR of an expert group meeting in December 2009 and v) an Indo-Swedish workshop held at New Delhi on 2nd February, 2010 to discuss on a joint strategy for containment of antimicrobial resistance (WHO, 2010).

Studies and reports on the emergence of drug resistant bacteria including MDR in both hospital-based and other environments from Kerala State are sparse with very few reports available in published literature (Manjusha and Sarita, 2011; Ahmed et al., 2012; Krishna et al., 2014) including the reports from the laboratory where the present study was carried out (Narayanan et al., 2016 and Nithya et al., 2017). Recently, Kerala government has formulated a new antibiotic stewardship programme to control antibiotic resistance, which not only takes stock of the antibiotic resistance in the state but also builds awareness among doctors, hospital staff and patients as well as the larger community on this critical health issue of antibiotic resistance (IIMR, 2016). A detailed survey of available literature including internet resources failed to reveal a clear picture on the extent or prevalence of antibiotic resistance mediated by efflux pump - based mechanisms and role of porin down-regulation despite several studies carried out across the world and a few from within the Indian subcontinent. Hence, the doctoral work presented in this Thesis, though limited, focuses on the above mentioned aspects of antimicrobial resistance mechanisms.

1.6 About the Thesis

1.6.1 Aims and objectives

The present study was undertaken

(i) to determine the prevalence of MDR gram-negative clinical bacterial isolates in Kerala

(ii) to gain deeper molecular insights into efflux pump and porin related genes in *P. aeruginosa* selected from clinical isolates of MDR gram-negative bacteria.

Objectives of this study included :

1. Collection of MDR gram-negative bacterial isolates from various clinical laboratories in Kerala

2. Identification of bacteria, antibiotic profiling and phenotypic detection of efflux pump - mediated drug resistance therein

3. Phenotypic detection of various beta-lactamases in *P. aeruginosa* selected from clinical isolates of MDR gram-negative bacteria and their RAPD profiling

4. Expression analysis of efflux pump and porin genes in the selected isolates of MDR *P. aeruginosa*

5. Mutational variations and phylogenetic analyses of efflux pump regulatory genes from MDR *P. aeruginosa*

6. Mutational variations and phylogenetic analysis of porin *oprD* gene, molecular modelling of porin protein and

7. Restriction mapping of *oprD*-derived amplicons for potential diagnostics.

1.6.2 Work plan and parameters evaluated in the present study

A flow chart summarizing the work plan and the parameters evaluated in the study is given below (Fig. 1.1) **:**

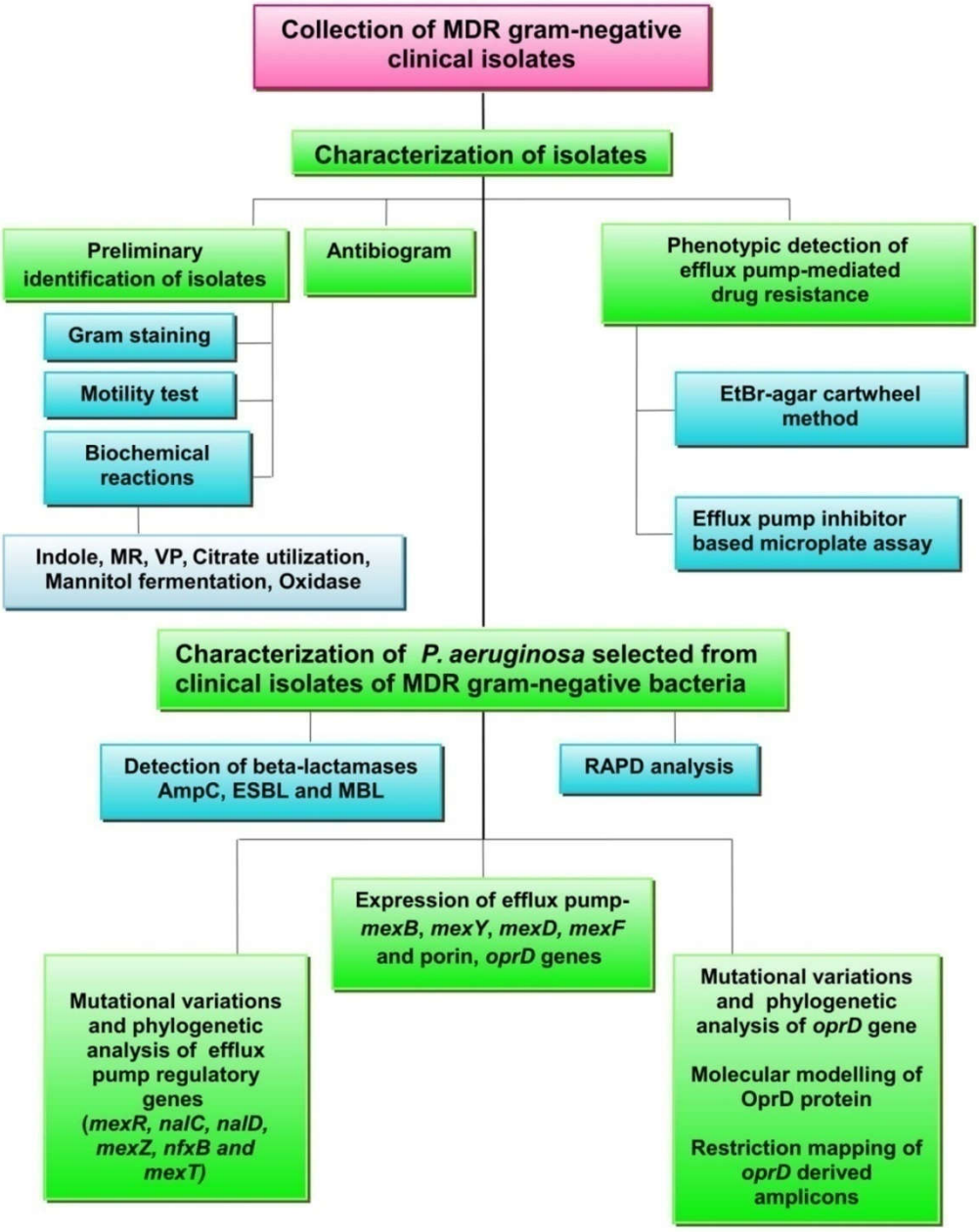


Fig. 1.1. Work plan and the parameters (flowchart) evaluated in the study.

1.6.3 Thesis layout

The Thesis includes six major chapters. The first gives a brief introduction on the major theme of the doctoral research work. The second chapter deals with review of literature which covers the discovery and classification of antibiotics, antibiotic resistance mechanisms, factors contributing to the spread of antibiotic resistance along with a general account on the present global and Indian scenario of antibiotic resistance. Details of the 'Materials and Methods' used in the study form the third chapter whilst the Fourth chapter incorporates the experimental results (presented in tables and figures), their analyses followed by relevant and appropriate discussions. The final and the fifth chapter consists of 'Conclusions' and 'Future prospectives'. This chapter summarizes the highlights of the entire research work and also provides a brief perspective on future directions and implications of the present study. The 'References' section gives full bibliographic information of all citations quoted in the text. An 'Appendix' given at the end contains information on reagents, solutions and buffers used in the study. *Pre-pages* (v - x) show lists of all abbreviations, figures and tables included in the Thesis.

REVIEW OF LITERATURE

2.1 Discovery of Antibiotics

The discovery of weapon against bacterial diseases was one of the most significant medical achievements of the 20th century. The beginning of modern 'antibiotic era' is usually associated with pioneer workers - Paul Ehrlich and Alexander Fleming. The idea of a 'magic bullet' to target bacterial pathogens led Ehrlich and his team discover, in 1909, the first antimicrobial agent 'Salvarsan', an arsenic-based chemical which proved to be an effective remedy for syphilis (Bosch and Laia, 2008). In 1935, Gerhard Domagk discovered that a synthetic red dye, a forerunner of sulfonamide, was effective in the therapeutics and management of bacterial infections which was later known commercially as Prontosil (Lesch, 2007). In 1928, Fleming serendipitously discovered the first natural antibiotic, penicillin, which was introduced into the market in 1945. Penicillin was an outstanding agent in terms of safety and efficacy and this drug saved the lives of many wounded soldiers during World War II (Saga and Yamaguchi, 2009; Aminov, 2010). The subsequent two decades became the 'Golden Age' of antibiotic discovery, both synthetic and naturally-occurring, including aminoglycoside, chloramphenicol, tetracycline, macrolide, glycopeptide and quinolone (Saga and Yamaguchi, 2009). Parallel to antimicrobial discovery, microbial resistance against antibiotics was also becoming evident, but modifications to the existing antibiotics, however, could produce active analogues to combat this obstacle (Lewis, 2013; Gould, 2016). A timeline of antibiotic discovery, deployment and emergence of antimicrobial resistance towards them is shown in Fig. 2.1. Evidently, most of the antibiotics were discovered only up to the 1970s period. The subsequent years witnessed the introduction of new functional groups for expansion of the existing scaffold with only a few new ones discovered in the past half-century (Das et al., 2017).

2.2 Antibiotic classification

Antibiotics are mainly classified according to their principal mechanism of action (Fig. 2.2, Table 2.1) such as inhibition of cell wall, protein, nucleic acid synthesis and metabolic pathway(s) besides disruption of cell-membrane function (Tenover, 2006; Džidić et al., 2008; van Hoek et al., 2011; Fernandes et al., 2013; Lewis, 2013; Liwa and Jaka, 2015).

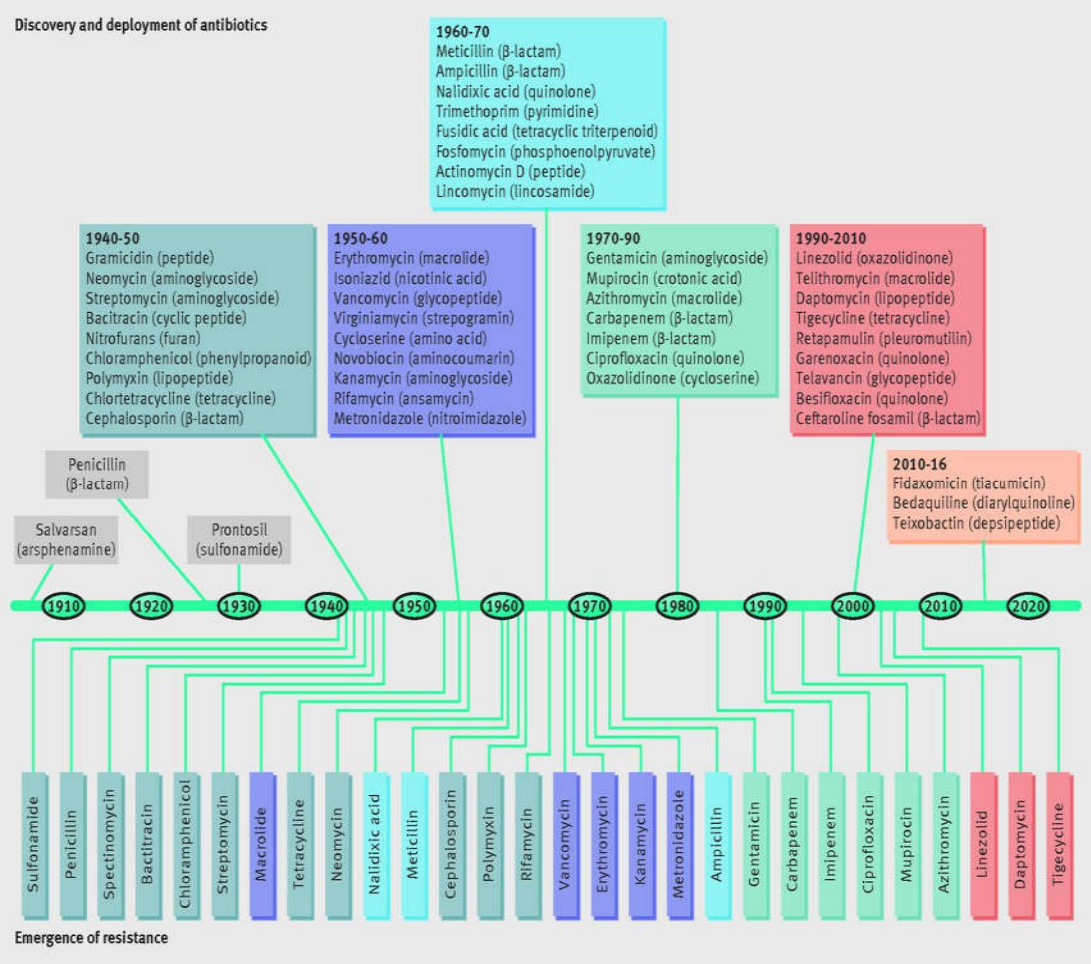


Fig. 2.1. Timeline of discovery, deployment of antibiotics in clinical practice and emergence of antimicrobial resistance (Das et al., 2017)

Table 2.1 Classification of antibiotics

Mode of Action	Class	Example
Interference with cell wall synthesis		
-binds to penicillin-binding protein	**Beta- lactams**	
	Penicillin and its derivatives	Penicillin G, Penicillin V, Methicillin, Oxacillin, Cloxacillin, Dicloxacillin, Floxacillin, Ampicillin, Amoxicillin, Piperacillin
	Cephalosporins	
	I generation	Cephalothin, Cephapirin, Cefazolin, Cephalexin, Cephradine, Cefadroxil
	II generation	Cefamandole, Cefuroxime, Cefonicid, Ceforanid, Cefoxitin,Cefmetazole, Cefminox, Cefotetan
	III generation	Cefotaxime, Ceftizoxime, Ceftriaxone, Ceftazidime, Cefoperazone,Cefixime, Ceftibuten, Cefdinir
	IV generation	Cefepime, Cefpirome
	V generation	Ceftobiprole, Ceftaroline
	Monobactams	Aztreonam
	Carbapenems	Meropenem, Imipenem, Ertapenem, Doripenem
-binds to D-alanyl-D-alanine termini of peptidoglycan chain	**Glycopeptides**	Vancomycin, Teicoplanin
Inhibition of protein synthesis		
-binds to 30S ribosomal subunit	**Aminoglycosides**	Amikacin, Gentamicin, Kanamycin, Neomycin, Tobramycin.
-binds to 30S ribosomal subunit	**Tetracyclines**	Tetracycline, Doxycycline, Minocycline
-binds to 30S ribosomal subunit	**Glycyclines**	Tigecycline
-binds to 50S ribosomal subunit	**Macrolides**	Erythromycin, Azithromycin, Clarithromycin
-binds to 50S ribosomal subunit	**Chloramphenicol**	
-binds to 50S ribosomal subunit	**Oxazolidinones**	Linezolid
-binds to 50S ribosomal subunit	**Streptogramins**	Quinupristin, Dalfopristin
-binds to 50S ribosomal subunit	**Lincosamides**	Lincomycin, Clindamycin

-binds to bacterial isoleucyl-tRNA synthetase	**Mupirocin**	
Interference with nucleic acid synthesis -inhibits DNA synthesis	**Quinolones**	Nalidixic acid, Ciprofloxacin, Norfloxacin, Levofloxacin, Moxifloxacin, Gatifloxacin
-inhibits RNA synthesis	**Rifamycins**	Rifampicin
Inhibition of metabolic pathway(s) - binds to dihydropteroate synthase (DHPS) and prevents folic acid production	**Sulfonamides**	Sulfamethoxazole
-inhibits dihydrofolate reductase (DHFR) and prevents folic acid production	**Trimethophrim**	
Disruption of bacterial membrane structure -depolarizes cell membrane	**Lipopetides**	Daptomycin
-binds to the lipid moiety of the lipopolysaccharide	**Polymixins**	Polymixin B, Colistin

2.3 Antibiotic resistance

Bacteria can survive in the presence of antimicrobial agents due to the existence of intrinsic, acquired or adaptive resistance mechanisms. Intrinsic resistance is an inherent attribute of a particular species to antibiotic agents as a result of (i) impaired permeability of the bacterial envelope, (ii) efficient drug export systems, (iii) absence or presence of low affinity antimicrobial target or (iv) presence of enzymes which inhibit or destroy antibiotics (Galán et al., 2013). Acquired resistance (Fig 2.2) can result from (i) mutations in chromosomal genes including those of spontaneous type, acquisition of hypermutability and adaptive mutations, (ii) acquisition and dissemination of antibiotic resistance determinants by horizontal transfer on plasmids, transposons, insertion sequences (IS), integrons ,

bacteriophages and genomic islands through processes such as transformation, transduction and conjugation (Tenover, 2006; Alekshun and Levy, 2007; Džidić et al., 2008; Giedraitienė et al., 2011). Adaptive resistance is the ability of bacteria to alter gene or protein expression temporarily as a result of exposure to environmental triggers such as stress, nutrient conditions, state of growth and sub-inhibitory levels of antibiotics. Such resistance is transient in nature, non-transmissible to subsequent generations and reversible upon removal of the inducing condition (Fernández and Hancock, 2012).

A single bacterial strain may possess various types of resistance mechanisms depending on its conditions of survival. Bacterial antibiotic resistance mechanisms of biochemical nature include (i) antibiotic inactivation, (ii) changes in target site and (iii) efflux pumps and outer membrane (OM) permeability changes (Fig 2.2).

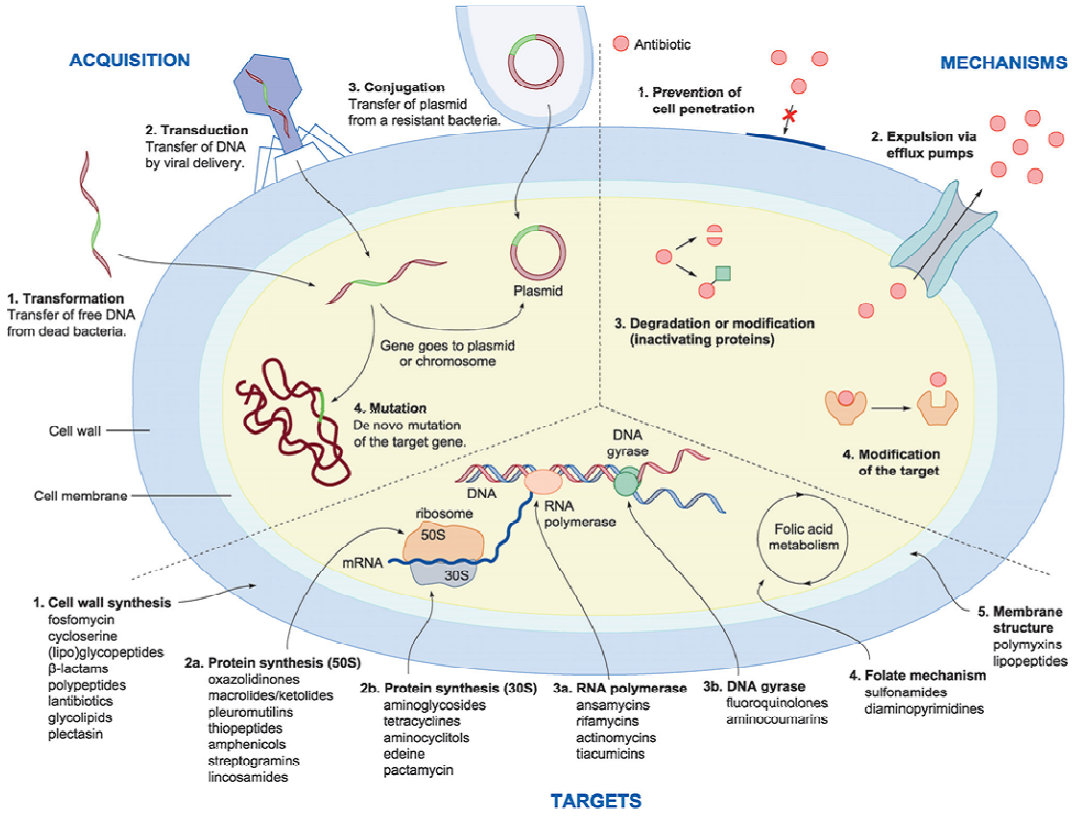


Fig. 2.2. Antibiotic resistance - pathways, mechanisms and targets in bacteria (Chellat et al., 2016).

2.4 Antibiotic inactivation

Bacteria can produce enzymes capable of degrading or modifying the antibiotic (s) by one or more of the following mechanisms:

2.4.1 Antibiotic inactivation by hydrolysis

Several enzymes are known to destroy antibiotic activity by cleaving the susceptible chemical bonds like esters and amides, before they reach their target. A classical example for amidases includes beta-lactamases that cleave the beta-lactam ring of the penicillin and cephalosporin antibiotics. The Ambler and the Bush-Jacoby-Medeiros are the two known classifications of beta-lactamases, where the Ambler classes are based on amino acid homology and the Bush-Jacoby-Medeiros classification is based on the substrates and inhibitors of the enzymes (Ambler, 1980; Bush and Jacoby, 2010). Extended spectrum beta-lactamases (ESBLs) are the mutant enzymes which hydrolyse penicillins, early and extended-spectrum cephalosporins, and aztreonam (but not cephamycins or carbapenems) and are susceptible to beta-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam. AmpC beta-lactamases hydrolyse broad and extended-spectrum cephalosporins but are not inhibited by beta-lactamase inhibitor (clavulanic acid). *Klebsiella pneumoniae* carbapenemase (KPC) enzymes are active against extended-spectrum cephalosporins, carbapenems and aztreonam. Metallo-beta-lactamases (MBLs) hydrolyse penicillins, cephalosporins, and carbapenems with the interaction of zinc ions in its active site and this is inhibited by EDTA. OXA (oxacillinase) group of beta-lactamases confer resistance to penicillins and carbapenems, except extended-spectrum cephalosporins (ceftazidime, ceftriaxone) and aztreonam (Fernandes et al., 2013; Munita and Arias, 2016). Other hydrolytic enzymes produced by bacteria include macrolide esterases and fosfomycin epoxidases. Erythromycin esterase encoded by *ereB* gene is prevalent in *Enterobacteriaceae* which inactivates erythromycin A and oleandomycin by hydrolyzing the lactone ring. Epoxidase hydrolyses reactive epoxide antibiotic, fosfomycin, through ring opening by either a thiol-containing co-substrate or water (Liwa and Jaka, 2015).

2.4.2 Antibiotic inactivation by group transfer

The group transferases belong to the most diverse family of resistant enzymes. Such enzymes inactivate and impair target binding of antibiotics like aminoglycosides, chloramphenicol, streptogramin, macrolides or rifampicin, by chemical substitution of adenylyl, phosphoryl or acetyl groups. All the modified chemical strategies including *O*-acetylation and *N*-acetylation, *O*-phosphorylation, *O*-nucleotidylation, *O*-ribosylation, *O*-glycosylation, and thiol transfer, require a co-substrate ATP, acetyl-CoA, NAD^+,UDP-glucose, or glutathione for their activity. Hence, these enzymes are active only in the cytoplasm (Wright, 2005).

2.4.3 Antibiotic inactivation by redox process

Antibiotic inactivation by the redox process has been occasionally exploited by pathogenic bacteria. There are a few examples of this strategy - oxidation of tetracycline antibiotics by the tetX enzyme and that of the protection of *Streptomyces virginiae* from endogenously produced type-A streptogramin antibiotic known as virginiamycin M1. In the latter case, this is achieved by reducing a critical ketone group into an alcohol at position 16 (Wright, 2005; Džidić et al., 2008).

2.5 Changes in target site

Bacteria can alter the molecular targets of antimicrobial agents which decrease its affinity or prevent the antibiotic from binding to its target site (Debabov, 2013; Blair et al., 2015; Munita and Arias, 2016) by one of the following means:

2.5.1 Target protection

Most of the clinically relevant genetic determinants coding for proteins that mediate target protection are carried by mobile genetic elements. Representative examples of this mechanism include, tetracycline resistant determinants Tet(M) and Tet(O) which act as homologues of elongation factors (EF-G and EF-Tu) in protein synthesis and interact with ribosome and dislodge tetracycline from its binding site.

Fluoroquinolone resistance determinant *Qnr* acts as a DNA homologue that competes for the DNA binding site of DNA gyrase and topoisomerase IV.

2.5.2 Target modification

Bacterial antibiotic resistance via target site modifications affects almost all families of antimicrobial compounds. These modifications include (i) point mutations in the genes encoding the target site, (ii) enzymatic alterations of the binding site (iii) replacement or bypass of the original target, finally resulting in decreased affinity for the target site.

Well-characterized examples of mutational resistance involve the mechanism of rifampicin, fluoroquinolone and oxazolidinones resistance. Rifampicin blocks bacterial transcription by binding to a highly conserved structure located in the beta-subunit of the DNA-dependent RNA polymerase which is encoded by *rpoB* gene. Mutations in the gene decreases affinity of the drug for its target thereby allowing transcription. Fluoroquinolone inhibits DNA replication by binding to DNA gyrase and topoisomerase IV. Mutations in the quinolone resistance determining region (QRDR) of the structural genes that sufficiently alter these enzymes result in less efficient binding of antibiotics. Oxazolidinones inhibit the formation of the initiation complex and interfere with translocation of peptidyl-tRNA from the A site to the P site. Mechanisms of resistance include mutations in the 23S rRNA resulting in decreased affinity for binding.

Target site modification by enzymatic alteration is exemplified by macrolide resistance mediated by the *erm* gene (erythromycin ribosomal methylation) encoded ribosomal methylase enzyme. The enzyme methylates 23S rRNA and alters the drug-binding site. Since macrolides, lincosamides, and streptogramin B antibiotics have overlapping binding sites in the 23S rRNA, methylation on these sites also result in cross-resistance to the lincosamide and streptogramin B class of antibiotics.

Bacteria can acquire genes homologous to the original target that carry out similar biochemical functions but are not inhibited by the antimicrobial molecule. In methicillin-resistant *Staphylococcus aureus* (MRSA), resistance is conferred by

acquisition of the staphylococcal cassette chromosome *mec* (SCC*mec*) element with three genes *mecRl-metf-mecA*. The *mecA* gene is responsible for methicillin resistance, which encodes penicillin binding protein- PBP2a, a bifunctional transglycosylase/transpeptidase with reduced affinity to beta-lactams. Some resistant bacteria can avoid antimicrobial action by bypassing the metabolic pathway they inhibit by overproducing the antibiotic target. Many trimethoprim (TMP) and sulfamethoxazole (SMX) resistant bacteria show this type of resistance mechanism. Trimethoprim and sulfonamide prevent folic acid production by inhibiting DHFR and DHPS respectively. In such bacteria, mutations occur in the promoter region of the DNA encoding DHFR or DHPS enzymes which leads to overproduction of these enzymes, prevent the ability of TMP-SMX to inhibit folate production thereby permitting bacterial survival.

2.6 Reduced uptake and active efflux

Reduced uptake and active efflux are the bacterial strategies for drug resistance that limit the intracellular access of antibiotics. Notably, alterations that restrict influx of antibiotics through outer membrane act in synergy with increased levels of antibiotic efflux (Fernández and Hancock, 2012).

2.6.1 Reduced permeability

The OM of gram-negative bacteria is an asymmetric bilayer of phospholipids and glycolipids mainly lipopolysaccharides (LPS) embedded with nonspecific porins and specific uptake channels (Zgurskaya et al., 2015). By modifying its lipid or protein composition, OM provides the first line of protection to the organism, without compromising the exchange of materials required for their survival.

2.6.1.1 Lipid-mediated resistance

LPS is composed of three components: (i) hydrophobic fatty acid chain bearing lipid A, a glucosamine disaccharide (ii) a heterogeneous core polysaccharide which is branched and consists of 6-10 sugars and two 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) and (iii) highly variable O antigen with 1-40 repeating units. The core polysaccharide is covalently bound to the lipid A through an acidic sugar,

Kdo. Many hydrophobic antibiotics gain access to the cell interior by diffusion through the lipid components of the outer membrane, but the core region of LPS acts as a barrier to hydrophobic antibiotics and other compounds and thereby provide intrinsic resistance (Delcour, 2009; Miller, 2016).

2.6.1.2 Porin-mediated resistance

Porins are membrane proteins which form water-filled pores with various grades of selectivity. They consist of transmembrane antiparallel beta-strands with alternating hydrophobic and hydrophilic amino acids facing outwards and inwards respectively and connected by short periplasmic turns and longer surface-exposed extracellular loops. They also act as molecular filters for hydrophilic substances and mediate transport of nutrients and ions across the membrane into the periplasm (Fernández and Hancock, 2012; Galdiero et al., 2012). Mechanisms of porin-mediated antibiotic resistance are achieved by three general processes : (i) shift of one or two major porins by another (ii) altered function due to specific mutations and (iii) change in porin expression levels, including loss or severe reduction. Resistance acquired through loss or functional changes of porins are reported in organisms like *Escherichia coli*, *P. aeruginosa*, *Neisseria gonorrhoeae*, *Enterobacter aerogenes, Enterobacter cloacae, K. pneumonia, Klebsiella oxytoca, Serratia marcescens*, *Salmonella enterica, Vibrio cholera* and *Acinetobacter baumannii*. Classical examples involving such resistance in major proteins produced by *E. coli* - OmpF, OmpC and PhoE, *P. aeruginosa* - OprD (protein D2) and *K. pneumonia* - OmpK35, OmpK36, have been included in Table 2.2 (Delcour, 2009; Fernández and Hancock, 2012; Munita and Arias, 2016).

Table 2.2 Porins related to antibiotic resistance in different bacterial species

Bacteria	Porin	Antibiotics
P. aeruginosa	OprD	carbapenems
E. coli	OmpC, OmpF, PhoE	beta-lactams
S. marcescens	OmpC, OmpF	beta-lactams
K. pneumoniae	OmpK35 OmpK36	cephalosporins,carbapenems, fluoroquinolones and chloramphenicol carbapenems
E. cloacae	OmpF	carbapenems
E. aerogenes	OmpC , OmpF Omp36	carbapenems imipenem, cefepime, and cefpirome
K.oxytoca	OmpK36	carbapenems
N. gonorrhoeae	PIB	beta-lactams, tetracycline and fluoroquinolones
S. enterica	OmpC OmpF	cephalosporins chloramphenicol and imipenem
V. cholerae	OmpU	Cephalosporins
A.baumannii	CarO	Carbapenems

2.6.1.2.1 *P. aeruginosa*: OprD-mediated antibiotic resistance

The *P. aeruginosa* porin-OprD is a 45-49 kDa substrate-specific outer membrane protein that facilitates the diffusion of basic amino acids, small peptides and carbapenems into the cell and also functions as a serine protease (Li et al., 2012). DNA and amino acid sequence identities of OprD range from 91 to 93% and 88 to 93% respectively among individual strains of *P. aeruginosa*. Despite the genetic variability of OprD among different strains, this aqueous porin shares close homology to the nonspecific *E. coli* porin OmpF (Pirnay et al., 2002; Lister et al., 2009). In 2007, X-ray crystal structure of OprD was determined by Biswas et al (Fig 2.3). The OprD crystallizes as a monomeric 18-stranded beta-barrel comprising 9 loops forming an outer membrane-spanning channel with a positively charged basic ladder on one side and an electronegative pocket on the other side. The presence of the two short beta-strands S5 and S6 in the OprD structure suggests that the latter may exist as a labile trimer in the outer membrane. The interior of the barrel consist of two long loops, L3 and L7, which fold inward to form a very narrow, roughly circular constriction with a diameter of ~5.5 A° (Biswas et al., 2007). The external loops 2 and 3 serve as binding sites for specific substrates like

basic amino acids and imipenem, and serve as a passage channel within OprD. Mutation or deletion within loops 2 and 3 result in conformational changes of OprD causing imipenem resistance. Deletion of loops 5, 7 and 8 lead to enhanced susceptibility to beta-lactams, quinolones other than fluoroquinolones, chloramphenicol and tetracycline, revealing that these three loops are involved in restricting the intracellular accumulation of certain antibiotics. Also, it was found that amino acid substitutions within loop 7 subsequently increased meropenem susceptibility (Huang and Hancock, 1996; Li et al., 2012).

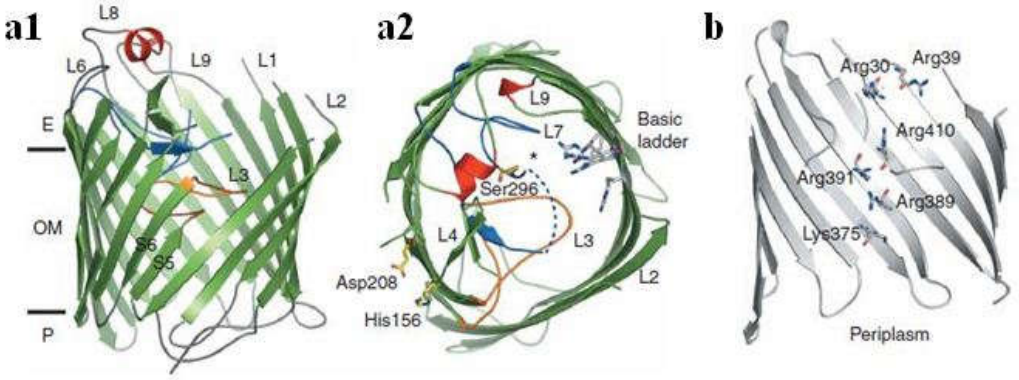


Fig. 2.3. Structure of P. aeruginosa OprD. *Cartoons of OprD:* ***a1****, viewed from the side and* ***a2,*** *from extracellular environment. Colors denote : green - beta-strands, gray - loops and turns, red – alpha helices, orange and blue - pore-constricting loops L3 and L7 respectively. E, extracellular space; OM, outer membrane; P, periplasmic space; dotted line represents the segment of loop L7 and* ***b****) Side view of the pore showing the basic ladder (Biswas et al., 2007).*

It is imperative to note here that the structural domains of the porin protein of the reference *P. aeruginosa* strain PAO1, according to the different published reports harbor contradictions. The structural representation of the channel protein based on amino acid sequence alignments or crystallographic data do not appear fully congruent essentially with regard to the number and positions of the loops. For instance, whilst Epp et al. (2001) and Sanbongi et al. (2009) show the presence of 8 loops based on amino acid sequence alignments, Biswas et al (2007), however, report the presence of 9 loops based on crystallographic data. Kos et al (2016), also

show the 9th loop, but the positions of almost all loops within the channel protein differ from the earlier versions mentioned.

Expression of OprD is linked to both carbon and nitrogen metabolism of *P. aeruginosa* and it is induced by arginine (mediated through regulatory protein ArgR), histidine, glutamate, or alanine (Ochs et al., 1999). OprD-mediated resistance pathway can involve mechanisms that decrease the transcriptional expression of *oprD* and/or mutations that disrupt the translational production of a functional porin. OprD transcription can interfere by characterized mechanisms including (i) disruptions of the *oprD* promoter as a result of deletions or insertions of IS elements within the upstream region of *oprD*. (ii) premature termination of transcription, (iii) negative regulation-mediated through the regulatory proteins CzcR and CopR in presence of zinc and copper, respectively, (iv) salicylate-mediated repression, (v) negative regulation by MexT, a regulatory protein of MexEF-OprN efflux pump, and (vi) repression by two-component regulatory system playing critical roles in multidrug resistance named ParR-ParS (Fig. 2.4). Mechanisms of OprD deficiency at translation level include (i) frameshifts and premature stop codons due to mutations, insertions, and/or deletions, and (ii) disruption of the structural gene by insertion of large IS elements (Lister et al., 2009).

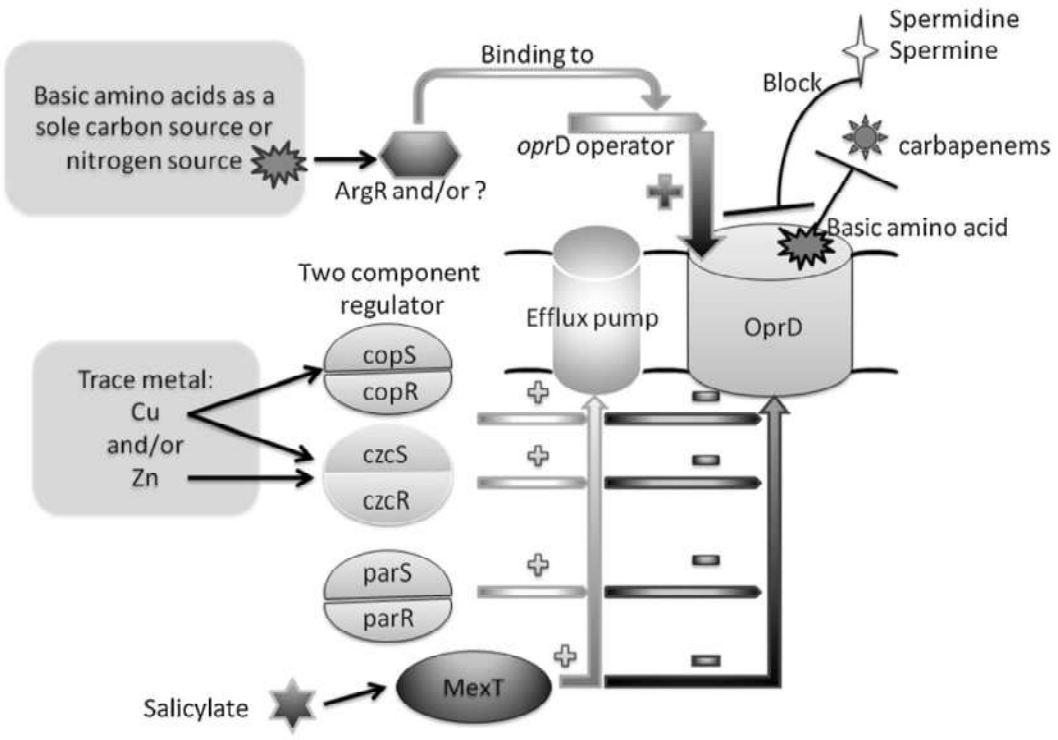


Fig. 2.4. Regulation of OprD expression and factors affecting carbapenem penetration into bacteria (Li et al., 2012).

2.6.2 Active efflux

Efflux systems are transport proteins which pump out toxic substrates including almost all classes of clinically relevant antibiotics from bacteria in an energy-dependent manner, without alteration or degradation of the drugs. Efflux pumps may be specific for one substrate or may transport a range of structurally unrelated compounds including antibiotics of multiple classes and can be associated with MDR (Webber and Piddock, 2003; Fernández and Hancock, 2012). Generally, antibiotic-specific efflux pumps are harboured on mobile elements, like transmissible plasmids and transposons, while MDR pumps are chromosomally encoded (Lomovskaya and Watkins, 2001). Multidrug transporters contribute significantly to bacterial intrinsic and acquired resistance. Although drug efflux systems are found in gram-negative and gram-positive bacteria, efflux-mediated resistance is more complex in gram-negative bacteria due to the molecular

architecture of the cell envelope. Efflux transporters may consist of single or multiple-component pumps. Efflux systems in gram-positive bacteria always comprise a single-component pump which transports their substrates across the cytoplasmic membrane. However, in gram-negative bacteria, many pumps have a tripartite organization to efflux substrates across the entire cell envelope (Moreira et al. 2004; Kumar and Schweizer, 2005). Bacterial efflux pumps are classified into various families based on their amino acid sequence homology, on the energy source that the pump uses, the number of components that the pump has (single or multiple), the number of transmembrane-spanning regions and the types of substrates that the pump exports (Fig 2.5). Families of transporter proteins include, (i) the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily, (ii) the major facilitator superfamily (MFS), (iii) the small multidrug resistance (SMR) family, (iv) the multidrug and toxic compound extrusion (MATE) family and (v) the resistance-nodulation-cell division (RND) superfamily (Piddock, 2006a, b). Recently, a sixth MDR family was identified and designated as proteobacterial antimicrobial compound efflux (PACE).

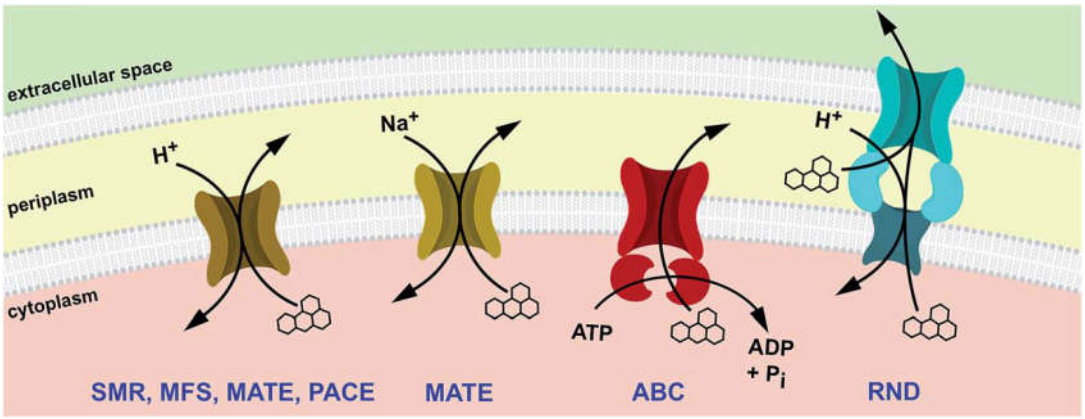


Fig. 2.5. Schematic illustration of efflux pump families (Mousa and Bruner, 2016).

2.6.2.1 ABC transporters

ABC efflux pumps are the members of primary active transporters found in both prokaryotes and eukaryotes. They utilize ATP hydrolysis for the export of a

wide array of substrates. The ABC superfamily consists of two highly conserved nucleotide-binding domains (NBDs) which bind and hydrolyze ATP and two highly diverse transmembrane domains (TMDs) containing 6 transmembrane helices. Sav1866 from *S. aureus* which served as the model for the study of ABC-mediated multidrug efflux mechanism is a structural homologue of the human MDR P-glycoprotein. Other ABC transporters have been listed as LmrA and LmrCD from *Lactococcus lactis*, BmrA protein from *Bacillus subtilis*, SmdAB of *S. marcesens*, EfrAB pump from *Enterococcus faecalis*, PatAB from *Streptococcus pneumoniae*, and MsbA from *E. coli* (Lewis et al. 2012; Du et al., 2015; Spengler et al., 2017).

2.6.2.2 MFS transporters

MFS is the largest known superfamily of secondary transporters which are widely distributed in both gram-positive and gram-negative bacteria. Efflux proteins transport structurally diverse compounds such as ions, sugars, amino acids, nucleic acids, intermediary metabolites, phosphate esters, oligosaccharides, antimicrobial agents (such as tetracycline, fluoroquinolone) and other small molecules across membranes via facilitated diffusion, symport, or antiport. The MFS transporters have 12 or 14 transmembrane-spanning segments (TMS) and most of their members are 400–600 amino acid residues in length. In gram-positive bacteria multidrug efflux members of the MFS include NorA, QacA, QacB, MdeA and SdrM of *S. aureus*, PmrA from *S. pneumoniae*, LmrP of *L. lactis*, whilst gram-negative bacteria can form tripartite efflux pump systems with an adaptor and an outer membrane protein, for example EmrAB-TolC of *E. coli* (Ranaweera et al., 2015; Spengler et al., 2017).

2.6.2.3 SMR and PACE transporters

The SMR transporters are the smallest known pumps which belong to the drug/metabolite transporter (DMT) superfamily and are energized by the proton-motive force. These proteins are ~ 110 amino acids in length and contain 4 TMS. Some of the examples for the characterized pumps of SMR family which export dyes, drugs and cations include the Smr pump of *S. aureus* and the EmrE pump of *E. coli* (Kumar and Schweizer, 2005; Li et al., 2015).

The PACE multidrug efflux system is the latest of the bacterial transporters to be described. This was identified in AceI (*Acinetobacter* chlorhexidine efflux) protein from *A. baumannii*, which shows resistance to the biocide chlorhexidine by active efflux mechanism. Also, many of the AceI homologs display resistance to chlorhexidine, benzalkonium, dequalinium, proflavine and acriflavine (Hassan et al., 2015; Mousa and Bruner, 2016).

2.6.2.4 MATE transporters

The MATE family of transporters is 400-700 amino acids in length and consists of 12 alpha-helical TMS. Members of this family are capable of extruding compounds such as norfloxacin, chloramphenicol, ciprofloxacin, kanamycin, ampicillin, metformin, cimetidine, ethidium bromide, benzalkonium chloride, and acriflavine. They use a gradient of Na^+, H^+, or both as the energy source. The NorM proteins described in *Vibrio parahaemolyticus* are well-studied representatives of MATE transporters which use a Na^+-coupled gradient for extruding compounds. Other multi-drug transporters of the MATE family include PfMATE from *Pyrococcus furiosus*, DinF from *Bacillus halodurans*, MepA from *S. aureus*, YdhE from *E. coli*, HmrM from *H. influenzae*, PmpM from *P. aeruginosa*, CdeA from *Clostridium difficile* and AbeM from *A. baumannii* (Kumar et al., 2013; Li et al., 2015; Spengler et al., 2017).

2.6.2.5 RND transporters

RND family of efflux pumps are widely distributed among bacteria, archaea and eukaryotes. These are the most clinically significant transporters associated with both intrinsic and acquired antibiotic resistance in gram-negative bacteria (Blair and Piddock, 2009) [Table 2.3]. In gram-negative bacteria, RND efflux pump is essentially a tripartite system composed of a cytoplasmic membrane transporter, a membrane fusion protein (MFP) and an outer membrane protein (OMP) channel, that ensures transportation of antimicrobial agents to the extracellular environment by receiving energy from the proton-motive force (Poole, 2004; Symmons et al., 2009; Daury et al., 2016). RND transporter protein consists of 12 TMS, with two large periplasmic loops between TMS 1 and 2 and TMS 7 and 8. AcrAB–TolC of

E. coli (Fig 2.6) and the MexAB-OprM of *P.aeruginosa* are the best-studied members of RND pumps (Putman et al., 2000; Kumar and Schweizer, 2005). Such structural studies have revealed that the RND pumps capture their substrates directly from the periplasm or from the outer leaflet of the cytoplasmic membrane (Nikaido and Pagès, 2012).

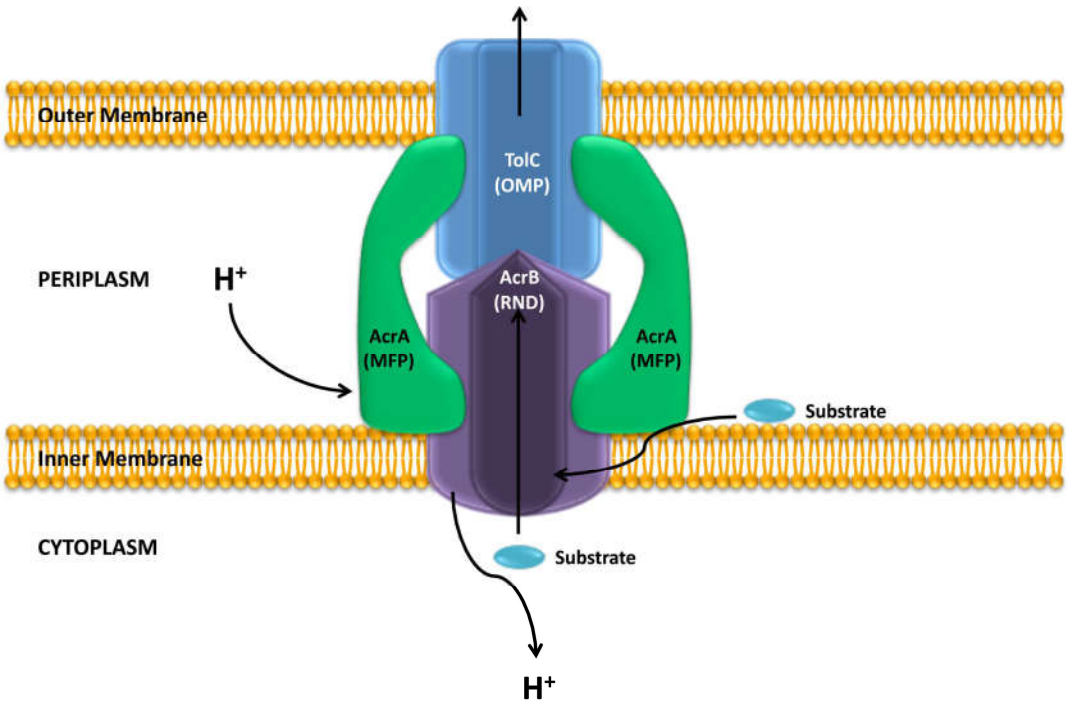


Fig. 2.6. Structure of E. coli RND efflux pump-AcrAB-TolC system (Alvarez-Ortega et al., 2013).

These transporters promote the extrusion of a wide range of compounds such as disinfectants, dyes, detergents, organic solvents, structurally unrelated anti-microbial agents and also play a role in virulence including colonization, evasion of host defense mechanisms, and biofilm formation (Fernando and Kumar, 2013). RND transporter is almost always chromosomally encoded and multiple pumps may occur in the same organism which often exhibit complex patterns of regulation independent of antibiotic exposure. Expression of RND encoding genes may also be triggered by ribosome disruption, the presence of reactive oxygen species, bile

salts, or other membrane damaging agents and stress conditions (Poole, 2008; Poole, 2012).

Table 2.3 Examples of RND efflux systems involved in antibiotic resistance

Bacteria	Efflux pump
A. baumannii	AdeABC, AdeFGH, AdeIJK
Aeromonas hydrophila	AheABC
Burkholderia cenocepacia	CeoAB-OpcM
Burkholderia pseudomallei	AmrAB-OprA, BpeAB-OprB, BpeEF-OprC
Campylobacter jejuni	CmeABC
E. aerogenes	AcrAB-TolC, EefABC
E. cloacae	AcrAB-TolC
E. coli	AcrAB-TolC, AcrAD-TolC, AcrEF-TolC, MdtABC-TolC, YhiUV-TolC
H. influenzae	AcrAB-TolC
K. pneumoniae	AcrAB
N. gonorrhoeae	MtrCDE
P. aeruginosa	MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY, MexPQ-OpmE, MexVW, MexMN, MexJK, TriABC-OpmB, MexGHI-OpmD, MuxABC-OpmB and CzcCBA.
Proteus mirabilis	AcrAB-TolC
S. marcescens	SdeAB, SdeCDE, SdeXY
Stenotrophomonas maltophilia	SmeDEF, SmeABC, SmeIJK, SmeYZ
*Salmonella t*yphimurium	AcrAB, MdtABC, MsdABC/TolC
V. cholerae	VexAB-TolC, VexEF-TolC

(*Fernández and Hancock, 2012; Sun et al., 2014*)

2.6.2.5.1 *P. aeruginosa*-RND transporters

A total of 12 RND efflux pumps (Table 2.4) have been recognized and identified in *P. aeruginosa* which include MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY, MexPQ-OpmE, MexVW, MexMN, MexJK, TriABC-OpmB, MexGHI-OpmD, MuxABC-OpmB, and CzcCBA (Lister et al., 2009; Li et al., 2015). Among such pumps MexAB-OprM, MexCD-OprJ, MexEF-OprN, and

MexXY contribute most significantly to antibiotic resistance (Dreier and Ruggerone, 2015). MexAB-OprM and MexXY are constitutively expressed in wild-type strains at a basal level and account for innate resistance. However, MexCD-OprJ and MexEF-OprN efflux pumps are induced by their substrates (Zavascki et al., 2010).

Table 2.4 Characteristics of RND efflux pumps in *P. aeruginosa*

Operon	Component	Regulator (s)	Substrate(s)	Reference
mexAB-oprM	MFP- MexA RND- MexB OMP- OprM	MexR, NalC, NalD	fluoroquinolones, beta-lactams, beta-lactamase inhibitors, tetracyclines, chloramphenicol, macrolides, novobiocin, trimethoprim, sulfonamides, carbapenems except imipenem, detergents, dyes, , biocides (triclosan), AHSLs, aromatic hydrocarbons	Lister et al., 2009; Li et al., 2015.
mexCD-oprJ	MFP- MexC RND- MexD OMP- OprJ	NfxB, EsrC	fluoroquinolones, beta-lactams, tetracycline, macrolides, trimethoprim, novobiocin, chloramphenicol, tigecycline, aromatic hydrocarbons, detergents, dyes, biocides (chlorhexidine, triclosan), organic solvents	Lister et al., 2009; Purssell and Poole, 2013; Purssell et al., 2015
mexEF-oprN	MFP- MexE RND- MexF OMP- OprN	MexT,MexS, MvaT	fluoroquinolones,carbapenem, chloramphenicol, trimethoprim, biocides (triclosan), aromatic hydrocarbons	Lister et al., 2009; Li et al., 2015
mexXY	MFP- MexX RND- MexY OMP- OprM/Opm-*	MexZ	fluoroquinolones, beta-lactams, tetracycline, aminoglycosides, macrolides, chloramphenicol	Lister et al., 2009; Morita et al. 2012b; Li et al., 2015
mexJK	MFP- MexJ RND- MexK OMP- OprM/OpmH	MexL	tetracycline, erythromycin , biocides (triclosan)	Lister et al., 2009; Li et al., 2015
mexGHI-opmD	MFP- MexH RND- MexI OMP- OpmD	SoxR	Fluoroquinolones, vanadium	Lister et al., 2009; Sakhtah et al., 2016
mexVW	MFP- MexV RND- MexW OMP- OprM		fluoroquinolones, tetracycline, chloramphenicol, erythromycin	Lister et al., 2009
mexPQ-opmE	MFP- MexP RND- MexQ OMP- OpmE		fluoroquinolones, tetracycline, chloramphenicol, macrolides	Lister et al., 2009

mexMN	MFP- MexM RND- MexN OMP- OprM		Chloramphenicol, fluoroquinolones, macrolides	Lister et al., 2009; Li et al., 2015
triABC	MFP- TriA MFP- TriB RND- TriC OMP- OpmH		Triclosan	Lister et al., 2009; Li et al., 2015
muxABC-opmB	MFP- MuxA RND- MuxB RND- MuxC OMP- OpmB		aztreonam, macrolides, novobiocin, tetracyclines	Mima et al., 2009
czcCBA	MFP - CzcB RND- CzcA OMP- CzcC	*CzcR-CzcS*	cadmium, cobalt and zinc	Hassan et al., 1999; Nies, 2003; Perron et al., 2004

* *MexXY may utilize OpmB, OpmG, OpmH, and/or OmpI as OMFs.*

2.6.2.5.1.1 MexAB-OprM

The MexAB-OprM pump was the first RND efflux system identified in *P. aeruginosa* (Poole et al., 1993). It displays wide substrate specificity, exports disinfectants, dyes, solvents, detergents, structurally unrelated antibiotics such as beta-lactams, beta-lactamase inhibitors, carbapenems except imipenem, fluoroquinolones, tetracyclines, chloramphenicol, macrolides, novobiocin, trimethoprim and sulfonamides (Li et al., 2015; Pourakbari et al., 2016). It also pumps out quorum-sensing mediators, acylhomoserine lactones (AHSLs), which induce the production of cell density-dependent virulence factors and quorum sensing inhibitors of nonnative *N*-acylated L-homoserine lactones (Minagawa et al., 2012; Moore et al., 2014).

Expression of the MexAB-OprM is governed mainly by regulatory loci such as *mexR*, *nalC* and *nalD* amongst several others (Fig. 2.7). The *mexR* gene, encoding a repressor protein of MarR family, is located upstream of the *mexAB-oprM* operon. An intergenic region with divergently oriented genes on either sides, *mexR* and *mexAB-oprM*, controls their transcription. MexR binds to this intergenic region as a stable homodimer and represses transcription from the *mexAB-oprM* operon. Mutations in *mexR* (*nalB* mutants), resulting in loss of dimerization and binding capacity of MexR protein, lead to hyperexpression of

MexAB-OprM. MexR protein is also endowed with oxidation-sensing mechanism which regulates virulence and antibiotic resistance in *P. aeruginosa* (Chen et al., 2008; Lister et al., 2009; Choudhury et al., 2016). The *nalC* encodes a protein, NalC, of TetR family which acts as a repressor of a divergent two-gene operon comprising of *PA3720* and *PA3719* (renamed *armR*). ArmR acts as an anti-repressor by allosterically inhibiting the dimeric MexR repressor resulting in derepression of *mexAB-oprM* (Cao et al., 2004; Braz et al., 2016). Another member of the TetR family of transcriptional regulators, NalD, acts as a secondary repressor of the tripartite MexAB-OprM multidrug efflux system by binding to a sequence between *mexAB-oprM* and the *mexR* binding site proximal to the *mexA* promoter. Hence, impairment of NalD in *nalD*-type mutants, leads to MexAB-OprM overexpression (Morita et al., 2006; Chen et al., 2016).

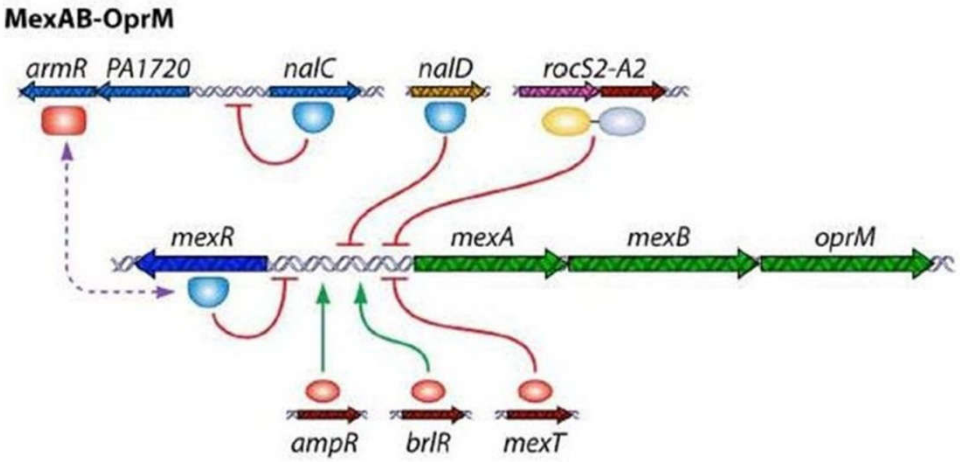


Fig. 2.7. Schematic diagram of transcriptional regulatory mechanisms controlling MexAB-OprM (Li et al., 2015).

It has been reported that MexAB-oprM expression reaches it maximum level at the onset of the stationary phase. At this phase, cell-to-cell quorum-sensing signal, *N*-3-oxo-dodecanoyl-L-homoserine lactone (3-oxo-C12-HSL) turns on and induces the expression of *mexAB-oprM* operon directly, independent of *mexR* gene. MexT, local activator of the MexEF-OprN system exerts a negative regulatory effect on MexAB-OprM expression through an uncharacterized mechanism in MexEF-OprN-overexpressed *nfxC* mutants (Maseda et al., 2004). RocA2, which is a

response regulator of pilus assembly machinery cluster *cupC* involved in adherence/ microcolony formation, exerts a negative regulatory effect on MexAB-OprM expression (Sivaneson et al., 2011). A biofilm-specific MerR-type regulator-BrlR, can activate MexAB-OprM expression by binding to promoter region of *mexAB-oprM* operon (Liao et al., 2013). By modulating expression of the MexR repressor, AmpR, a LysR-type regulator of AmpC beta-lactamase also can positively regulate MexAB-OprM efflux pump (Balasubramanian et al., 2012). Recently, Tian et al. (2016) reported that CpxR, a regulator of the cell envelope stress response in *E. coli* can directly involved in activation of MexAB-OprM in *P. aeruginosa*.

2.6.2.5.1.2 MexCD-OprJ

MexCD–OprJ pump normally quiescent in wild-type strain of *P. aeruginosa* does not contribute to intrinsic antimicrobial resistance. It can extrude a variety of antimicrobial agents, including fluoroquinolones, beta-lactams – preferentially, fourth-generation cephalosporins (cefepime, cefpirome, and cefozopran), chloramphenicol, tetracycline, novobiocin, trimethoprim, macrolides and tigecycline. It also exports biocides (triclosan and chlorhexidine), organic solvents, dyes and detergents (Lister et al., 2009; Purssell and Poole, 2013).

The *mexCD-oprJ* operon is regulated by repressors such as the NfxB and EsrC (Fig.2.8). The *nfxB* gene encoding repressor protein NfxB is located upstream of *mexCD-oprJ* but transcribed divergently from the operon. NfxB shows similarity to proteins of the LacI-GalR family. It binds to a site composed of two 39 bp repeats within the *nfxB-mexC* intergenic region and negatively regulates *mexCD-oprJ* as well as its own expression. Mutations within *nfxB gene* negatively impact NfxB repressor activity leading to hyperexpression of *mexCD-oprJ,* and strains harbouring such defects are known as *nfxB*-type mutants (Lister et al., 2009; Purssell and Poole, 2013). EsrC, a second regulator of MexCD-OprJ, is encoded by a gene *PA4596* which is located downstream of *mexCD-oprJ* operon. EsrC represses the transcription of *mexCD-oprJ* only under 'envelope stress' (membrane-damaged) condition and is dependent on NfxB (Purssell et al., 2015). MexCD–OprJ is

inducible in response to non-antibiotic compounds like benzalkonium chloride, chlorhexidine gluconate, tetraphenylphosphonium chloride, ethidium bromide, rhodamine 6G, and acriflavine. Agents such as chlorhexidine disrupt cell membrane leading to production of membrane lipid derivatives which stimulate membrane-associated Muc proteins. This in turn activates stress response sigma factor AlgU resulting in hyper-expression of MexCD-OprJ. Overexpression of the latter protein complex in *nfxB* type mutants is also dependent on AlgU (Morita et al., 2003; Fraud et al., 2008).

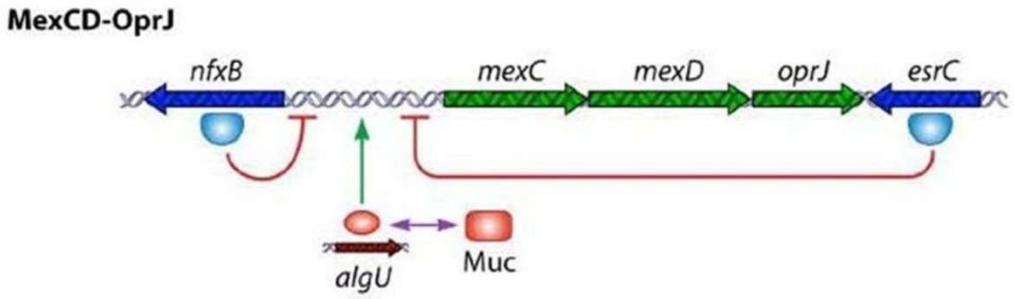


Fig. 2.8. Schematic diagram of transcriptional regulatory mechanisms controlling MexCD-OprJ (Li et al., 2015).

2.6.2.5.1.3 MexEF-OprN

MexEF-OprN is expressed at very low levels in wild-type and does not significantly contribute to intrinsic resistance against antibiotics. The *mexEF-oprN* operon is overexpressed in *nfxC*-type mutants of *P. aeruginosa* which confers resistance to fluoroquinolones, chloramphenicol, trimethoprim, and carbapenems (Köhler et al., 1997). This efflux pump is positively controlled by the transcriptional regulator MexT, a LysR family protein, encoded by *mexT* gene located upstream of the *mexEF-oprN* operon. MexT protein binds to the upstream nod box in *mexEF-oprN* promoter region and causes the hyperexpression of MexEF-OprN (Fig. 2.9). A concurrent downregulation of the outermembrane protein-OprD was reported in *nfxC*-type mutants leading to decreased imipenem susceptibility (Köhler et al., 1999). The *nfxC*-type mutants also show hypersusceptibility to various beta-lactams and aminoglycosides because of the reduced expression of *mexAB-oprM* (Maseda et

al., 2004). Interestingly, three types of *mexT* variations have been reported in wild-type strains and *nfxC* mutants, which possess inactive and active forms of MexT, respectively. Type I variation shows an additional mutation in *mexT* of *nfxC*-type mutant derived from wild type parent leads to the production of active MexT. In type II variation *mexT* gene in the *nfxC*-type mutants from wild type parent had a deletion of the 8-bp insert producing active form of MexT. In the case of type III variation, both the wild type strain and its *nfxC*-type derivative produced identical and active MexT (Maseda et al., 2000). The *nfxC* mutants with type III variations possess an additional mutation in *mexS* gene, located immediately upstream of *mexT* which encodes a putative oxidoreductase/dehydrogenase homologue. Inactivation of MexS protein eventually leads to intracellular accumulation of toxic metabolites that serve as effector molecules for MexT, which in turn upregulates *mexEF-oprN* expression to export the noxious intermediates (Sobel et al., 2005).

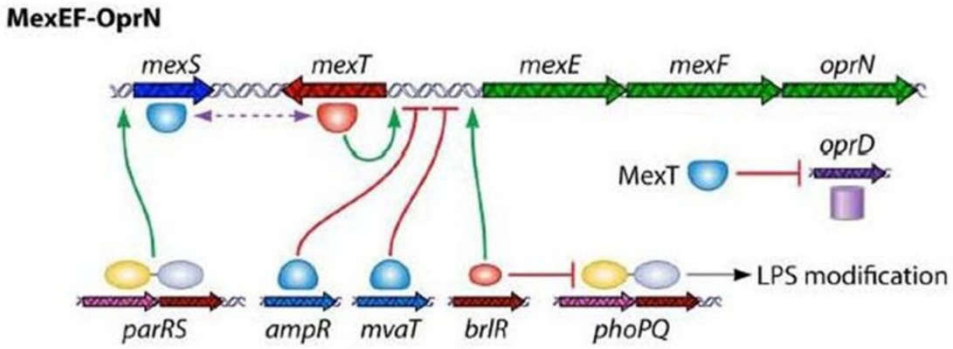


Fig. 2.9. Schematic diagram of transcriptional regulatory mechanisms controlling MexEF-OprN (Li et al., 2015).

Expression of *mexEF-oprN* is also controlled by histone-like nucleoid structuring family protein, MvaT, which acts as a global regulator affecting the expression of hundreds of genes including *mexEF-oprN,* biofilm formation, housekeeping, quorum sensing, and virulence. MvaT binds preferentially to AT-rich regions of DNA with a high affinity and silences the expression of certain genes. Inactivation or mutation of *mvaT* leads to MexEF-OprN hyperexpression independent of *mexT* or *mexS* and a reduction of OprD protein level (Westfall et al.,

2006; Castang et al., 2008). Mutations in the ParSR system downregulate the expression of both *mexS* and *mexEF-oprN* while genetic inactivation of AmpR leads to overexpression of MexEF-OprN with an MDR phenotype. BrlR regulator also plays a role in MexEF-OprN hypper-expression (Lister et al., 2009; Li et al., 2015).

2.6.2.5.1.4 MexXY

Unlike other operons encoding the MexAB–OprM, MexCD–OprJ and MexEF–OprN systems, *mexXY* operon lacks the gene encoding outermembrane protein. Instead, the MexXY system primarily utilizes OprM as its outer membrane component and possibly other outer membrane proteins, such as OpmB, OpmG, OpmH, and OpmI to form a functional tripartite (Lister et al., 2009). In MDR clinical isolate designate PA7 and its relatives, the *mexXY* operon contains gene encoding an OprM-like protein known as OprA, which is absent in other *P. aeruginosa* strains. OprA protein displays a higher level of sequence similarity with OM channels of *Burkholderia* efflux pumps than with those of *P. aeruginosa* (Roy et al., 2010; Morita et al., 2012a). The MexXY-OprM multidrug efflux system is considered as a significant determinant of aminoglycoside resistance in clinical strains of *P. aeruginosa*, particularly those isolated from the patients with cystic fibrosis. MexXY-OprM contributes to resistance towards a wide range of antibiotics such as cephalosporins (cefepime, cefpirome), macrolides (erythromycin), fluoroquinolones (ciprofloxacin), tetracyclines and tigecycline in addition to aminoglycosides (Morita et al., 2012b). Its contribution to intrinsic resistance is restricted to those antimicrobial agents like tetracycline, erythromycin, and gentamicin which are able to induce *mexXY* expression (Masuda et al., 2000).

Expression of the *mexXY operon* is negatively regulated by the *mexZ* gene product, located upstream of the operon and transcribed divergently (Fig. 2.10). Yet another member of TetR family of proteins, MexZ contains a DNA-binding helix–turn–helix motif at its N-terminal. Three types of MexXY-overproducing mutants have been described and they are classified as *agrZ, agrW1* and *agrW2*. The *agrZ* mutants harbour mutations inactivating *mexZ* gene including those caused by single amino acid substitutions in the DNA-binding, dimerization or other structural

domains of the encoded repressor. In *agrW1* mutants, impaired protein synthesis occurs due to a variety of defects in ribosomal proteins such as L1, L25, L21 and L27, or components of the Fmt bypass (methionyltRNA fMet formyltransferase FolD). It was reported that the induction of *mexXY by* protein synthesis inhibitors is dependent on ArmZ. The *armZ* (*PA5471*) transcription is also induced by the same ribosome inhibitors which induce *mexXY*. Induction of ArmZ indirectly or directly alters MexZ activity which results in *mexXY* upregulation. Activation of MexXY expression also occurs through AmgRS system regulating pathway where th *mexXY* expression occurs via its positive effect on the expression of the *htpX* (encoding an IM-associated protease) and PA5528 genes (encoding a modulator of FtsH protease). The *agrW2* mutants hyperexpress MexXY, with alterations in the sensor ParS or the response regulator, ParR - a two-component ParRS system - known to play critical roles in multidrug resistance. The ParRS system downregulates *oprD* with a concomitant upregulation of *mexXY* and the LPS modification operon, *arnBCADTEF-ugd*, which leads to active drug efflux and reduced OM permeability (Lister et al. 2009; Morita et al. 2012b; Li et al. 2015).

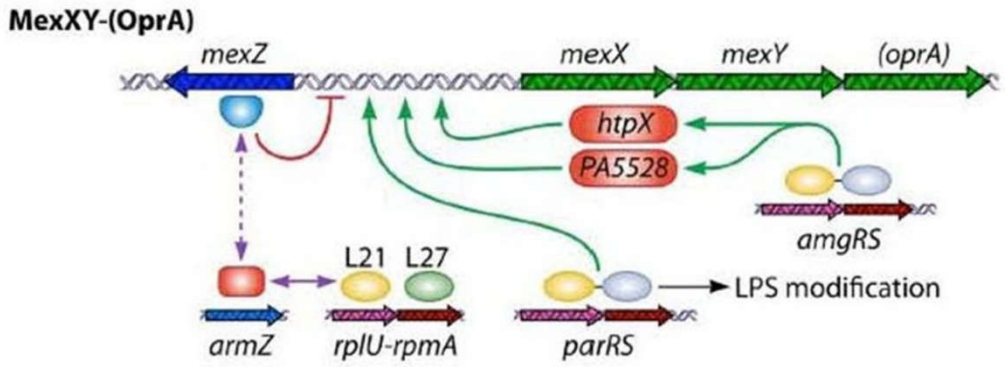


Fig. 2.10. Schematic diagram of transcriptional regulatory mechanisms controlling MexXY-(OprA) (Li et al., 2015).

2.7 Factors contributing to spread of antibiotic resistance

Antimicrobial selection pressure and spread of resistant organisms are the main leading factors for the emergence of resistance. Poor infection control, inadequate sanitary conditions, inappropriate food-handling, travel, indiscriminate use of antibiotics in medical-veterinary practice, communities, agriculture and aquaculture has encouraged the spread of antimicrobial resistance (Sharma, 2011; von Wintersdorff et al., 2016) [Fig. 2.11]. Activities such as indiscriminate antimicrobial use by physicians and the community, use of broad-spectrum antimicrobials, unskilled clinical practitioners, direct availability of medicines to patients without prescription, self medication, lack of frequent monitoring of antibiotic therapy, lack of awareness of the problem of antibiotic resistance in the general public, ultimately cause improper antibiotic dosage. These factors lead to exposure of antibiotics in sub-inhibitory or prolonged concentration resulting in the persistence of enough selective pressure for the development of antibiotic resistance by genetic alterations, such as changes in gene expression, horizontal gene transfer, and mutagenesis (Laxminarayan and Brown, 2001; Ventola, 2015).

Antibiotics used in agriculture have several different applications which can promote selection of resistant bacteria. These possibly affect humans by their release in the environment. Animals receive antibiotics in their food, water, or parenterally for the treatment of infectious diseases or non-therapeutic purposes such as growth promotion and prophylaxis, which may be responsible for carrying antibiotic resistant strains. Food chain as well as direct or indirect contact can be the route of transmission of antibiotic-resistant bacteria between animal and human populations (Paphitou, 2013; Zaman et al., 2017). The majority of consumed antibiotics are introduced into the environment through wastewater treatment plant effluents, hospital and processing plant effluents, application of agricultural waste and biosolids to fields, leakage from waste-storage containers and landfills, pharmaceutical waste and industrial effluent. Due to such antibiotic pollution, antibiotic resistance genes and mobile genetic elements can be co-released into the environment with sub-inhibitory concentrations of antibiotic compounds which can

provide stable selection pressure and favourable conditions for horizontal gene exchange and spread of resistance (Tello et al., 2012; Choudhury et al., 2012; von Wintersdorff et al., 2016).

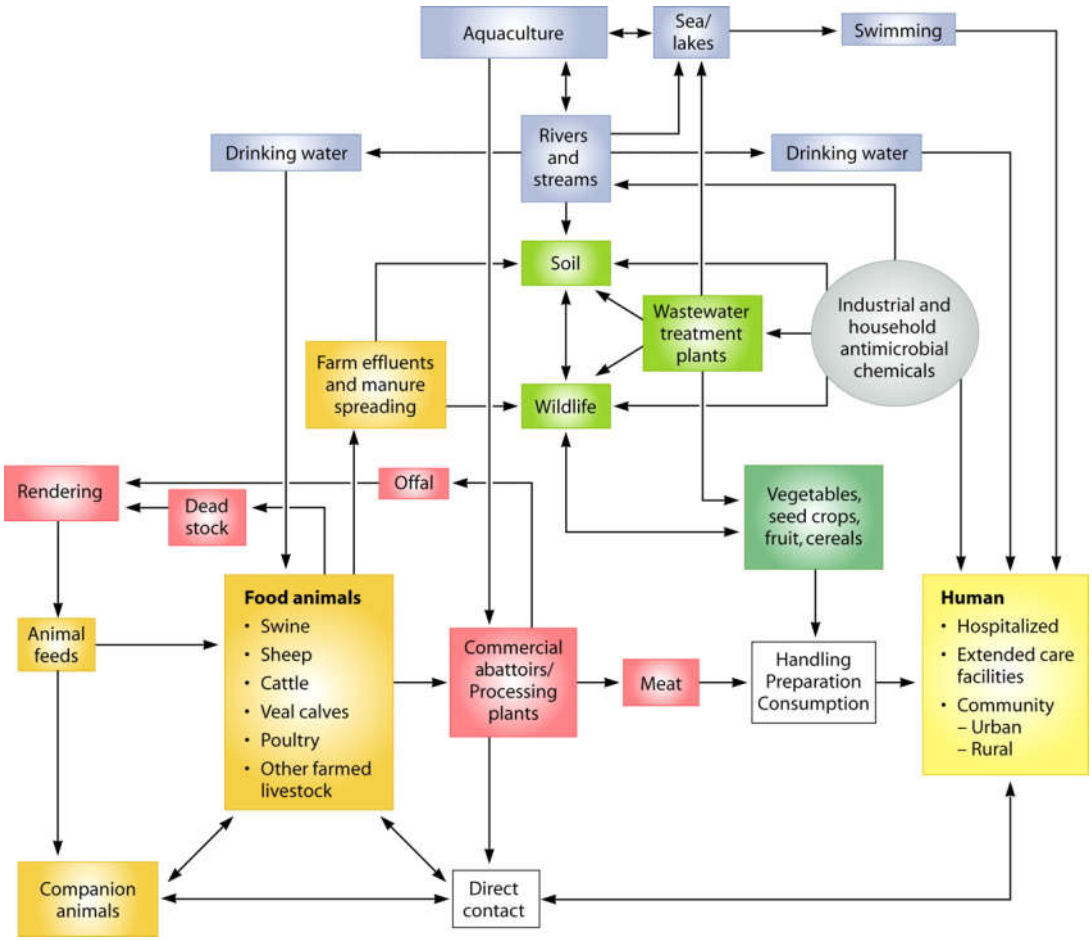


Fig. 2.11. Dissemination of antibiotics and antibiotic resistance (Davies and Davies, 2010).

2.8 Current global scenario of antimicrobial resistance

Antimicrobial resistance is an increasingly serious threat to global public health and therapy for common infectious diseases, leading to prolonged illness, disability, and death. WHO's first release of surveillance data on antibiotic resistance reveals that high levels of resistance towards a number of serious bacterial infections are present in both high and low-income countries. Notably, WHO's Global Antimicrobial Surveillance System (GLASS) has recently revealed the

widespread occurrence of antibiotic resistance among five lakh people with suspected bacterial infections across 22 countries (WHO, 2018a). Further, WHO has categorized 12 families of bacteria which pose a serious health threat to man as antibiotic-resistant "priority pathogens", as critical, high and medium priority according to the urgency of need for new antibiotics. The most critical group includes carbapenem-resistant *A. baumannii*, carbapenem-resistant *P. aeruginosa*, carbapenem-resistant, ESBL-producing *Enterobacteriaceae*, which pose health threat in hospitals, nursing homes, and among patients whose care requires devices such as ventilators and blood catheters. This critical group of bacteria can cause severe and often deadly infections such as bloodstream infections and pneumonia. High priority group comprise vancomycin-resistant *Enterococcus faecium*, methicillin-resistant/ vancomycin intermediate and resistant *S. aureus,* clarithromycin-resistant *H. pylori*, fluoroquinolone-resistant *Campylobacter* spp., fluoroquinolone-resistant *Salmonellae*, 3rd generation cephalosporin and fluoroquinolone-resistant *N. gonorrhoeae.* Third category of medium priority pathogens consist of penicillin-non-susceptible *S. pneumonia*, ampicillin-resistant *H. influenza* and fluoroquinolone-resistant *Shigella* spp (WHO, 2017a).

The *global rise* of carbapenem-resistant gram-negative bacteria over the past decade is *alarming* and is associated with high morbidity and mortality. Reports show that in United States, the overall disease burden and impact on survival was greatest among *A. baumannii* and *P. aeruginosa* followed by *K. pneumoniae* and *E. coli* (Cai et al., 2017). Studies from South Korea have reported that 32 -56% of patients hospitalized were infected with carbapenem resistant *Acinetobacter* spp. Among *P. aeruginosa* isolates, carbapenem resistance rates in most countries range from 10 to 50% (Gniadek et al., 2016). In 2015, the population-weighted mean percentage of carbapenem resistant isolates from Europe was 17.8% for *P. aeruginosa*, 8.1% for *K. pneumoniae* and 0.1% for *E. coli.* During the period 2012-2015, an increasing trend of carbapenem-resistance was observed in *K. pneumoniae* particularly in Croatia, Portugal, Romania and Spain (WHO, 2017b). In 2013, 17 out of 22 European countries reported 85-100 % prevalence of ESBL-

producing *E. coli.* During the period of 2009-2010, 28% of the *E. coli* from 11 Asian countries was reported to be ESBL producers (Chaudhary, 2016).

Globally, *Mycobacterium tuberculosis* is already considered as an established priority pathogen for which innovative new treatments are urgently needed. In 2016, as much as 45% of new tuberculosis (TB) cases reported were in Asia followed by 25% of them reported from Africa. Seven countries, namely, India, Indonesia, China, Philippines, Pakistan, Nigeria, and South Africa, account for 64% of the new TB cases. MDR-TB remains a public health crisis and there were six lakh new cases with resistance to the most effective first-line drug, rifampicin, with about 4.9 lakh cases of MDR type (WHO, 2018b). Among gram-positive bacteria, MRSA especially is considered as a widely recognized pathogen causing worldwide hospital and community acquired infections. More than 50% of MRSA hospital cases were reported from Asia, Malta, North and South America (Sit et al., 2017). The proportion of MRSA has declined in US, Europe, Canada and South Africa over the past few years. But in sub-Saharan Africa, Australia, Latin America and India, increased MRSA prevalence has been observed (Gelband et al., 2015). Emergence of vancomycin-intermediate *S.aureus* (VISA) and vancomycin resistant *S.aureus* (VRSA) has also been reported from various countries following introduction of vancomycin for MRSA treatment (Molton et al., 2013). Notably, vancomycin resistance in *E. faecium* showed a significantly increasing trend from 2011 to 2014, which remains a major infection control challenge. In case of *S. pneumoniae* percentages of resistance remained stable during the period 2011-2014, and it remained less susceptible to macrolides than penicillin (Chaudhary, 2016).

2.9 Current national scenario of antimicrobial resistance

Emergence of antimicrobial resistance is a major public health concern in India. Disease burden in India was among the highest in the world in 2010 with nearly 3.6 million episodes of severe pneumonia and 0.35 million pneumonia deaths reported in children less than 5 years of age (Farooqui et al., 2015). The Center for Disease Dynamics, Economics and Policy (CDDEP) report showed that occurrence of MRSA increased from 29% to 47% during the period of 2008-2014. According

to Indian Network for Surveillance of Antimicrobial Resistance (INSAR) reports, based on data from 15 tertiary care centres, there is incidence of 41% MRSA and a high rate of resistance towards ciprofloxacin, gentamicin, cotrimoxazole, erythromycin, and clindamycin. Another study reported occurrence of community acquired MRSA in 10% and reduced susceptibility to vancomycin in about 12% of *E. fecalis* isolates (WHO-Govt. of India Ministry of Health and Family Welfare Report, 2016; NAP-AMR, 2017). India continues to carry the largest estimated burden of TB including MDR-TB with nearly 1.3 lakh patients detected annually in the country including 79000 pulmonary MDR-TB patients. India accounts for the second highest number of estimated HIV-associated TB in the world. Around 1.1 lakh HIV-associated TB occurred in 2015 culminating in 37,000 deaths (TB India, 2017).

ESBL-producing *Enterobacteriaceae* have emerged as a challenge in India among both hospitalized patients and in the community. The 61% of ESBL-producing *E. coli,* 31–51% carbapenem-resistant *Klebsiella* species, 65% ceftazidime resistant *Pseudomonas* species and 42% imipenem-resistant *Pseudomonas* species were reported in the multicentric study conducted from seven tertiary care hospitals in Indian cities (NAP-AMR, 2017). Resistance to the broad-spectrum antibiotics such as fluoroquinolones and 3rd generation cephalosporins was observed in more than 70% bacteria including *A. baumannii*, *E. coli*, and *K. pneumoniae*, and above 50% in *P. aeruginosa* strains. Carbapenem resistance was reported in ~70% of A. *baumannii*, 57% of *K. pneumonia*, above 40% in *P. aeruginosa*, and more than 10% in *E. coli* (CDDEP. 2017). In India, plasmid-encoding carbapenem-resistant MBL (Singh, 2011; Nithya et al., 2017) earlier known as New Delhi metallo-beta-lactamase-1 (NDM-1) has been reported as the predominant gene encoding for carbapenem resistance in *Enterobacteriaceae* (Logan and Weinstein, 2017). Increasing use of colistin for treatment of carbapenem-resistant gram-negative bacterial infections has resulted in the emergence of colistin resistance which poses additional challenges for treatment and health management (Manohar et al., 2017).

MATERIALS AND METHODS

3.1 Bacterial isolates

MDR gram-negative bacterial isolates were collected from various clinical laboratories in Kerala during the period 2012 - 2016. Isolates were received after primary identification in pure line form as stab cultures. On reaching the laboratory, the isolates were then streaked on Luria Bertani (LB) and McConkey agar plates and their identities were reconfirmed by gram staining, motility testing and biochemical reactions essentially as described by Baron et al., 1994. Except *P.aeruginosa*, all other isolates were stored as glycerol stock cultures kept at -20ºC. *P.aeruginosa* isolates were maintained as stab cultures at 4ºC since they showed impaired revival capacity from glycerol stocks for reasons which continue to remain unclear. Microbial Type Culture Collection (MTCC) strains obtained from IMTECH (Chandigarh), India, used in this study included *P. aeruginosa* - PAO, *P. aeruginosa* -2453 and *E.coli* - 41.

3.2 Gram staining

Heat fixed smears of individual bacterial suspensions were prepared on clean glass slides. These slides were then flooded with primary stain, crystal violet solution and allowed to stain for a minute. The crystal violet was gently rinsed out with tap water and the smear was then flooded with the mordant, gram's iodine solution and incubated for a minute. The mordant was rinsed off with tap water and the smear was decolorized with alcohol or acetone until the blue colour disappeared completely. The slide was then rinsed with water and flooded with the counter stain, safranin for 30 s followed by a rinse with tap water. The air-dried preparation was then observed under oil immersion objective of microscope.

3.3 Biochemical reactions

3.3.1 Indole test: This test is used to determine the ability of an organism to produce tryptophanase enzyme. For this, tryptophan containing media was

inoculated with bacterial culture and incubated for 24 h. To this, 0.5ml of Kovac's reagent was added and gently shaken. A pink to wine colored ring at the interface between the broth and reagent indicated tryptophan degradation.

3.3.2 Methyl red (MR) test: Ability of an organism to produce and maintain stable acid end products from glucose fermentation was determined by MR test. To 5.0 ml of bacterial suspension, 5 drops of MR reagent was added. A bright red colour was indicative of acids produced by mixed acid fermentation of glucose.

3.3.3 Voges-proskauer (VP) test: This test detects the ability of bacteria to convert the acid products to acetoin and 2,3-butanediol. To 2.0 ml of bacterial suspension, 12 drops of VP reagent-A was added followed by 4 drops of VP reagent-B. The mixture was then shaken and incubated at room temperature for 30 min. A red colour indicated acetoin production.

3.3.4 Citrate utilization test: This test determines the ability of bacteria to utilize sodium citrate and inorganic ammonium salts as the sole sources of carbon and nitrogen respectively. Bacterial suspensions were inoculated onto the slant agar containing Simmon's citrate medium and incubated at 37ºC for 24 h. Bacterial growth followed by a change in colour from green to blue indicated the presence of alkaline end products of citrate utilization.

3.3.5 Mannitol motility test: Bacterial stab cultures were prepared in semi-solid, mannitol motility media and incubated at 37ºC for 24 h. The presence of motile bacteria was observed as diffused growth throughout the medium in comparison to growth only along the line of inoculation in the case of non-motile bacteria. A change in the colour of the medium from red to yellow indicated mannitol fermentation.

3.3.6 Oxidase test: This test determines the presence of cytochrome oxidase activity in bacteria. Using wooden or platinum wire loop, a bacterial colony was taken and smeared on a filter paper soaked with the substrate - tetramethyl-p-phenylenediamine dihydrocholride. A colour change of the inoculated area to deep

blue or purple within 10 s due to the production of indophenol indicated a positive result.

3.4 Antimicrobial susceptibility testing

Antibiotic sensitivity test was done by standard disc diffusion method (Kirby-Bauer Method) on Mueller–Hinton agar (MHA) plates. For the test, 3-5 isolated colonies were selected from an agar plate culture, inoculated into LB broth and incubated at 37ºC. The density of bacterial suspension was compared and adjusted to 0.5 McFarland standard (1.5 x 10^8 CFU/ml). A sterile cotton swab was soaked in the bacterial culture and swabbed on MHA plates to generate a bacterial lawn. Pre-determined batteries of antimicrobial discs (purchased from Himedia, Mumbai, India) were placed over the lawn culture and incubated for 24 h at 37ºC. The zone diameters were recorded and interpreted as sensitive, intermediate and resistant as per the Kirby-Bauer chart (CLSI, 2012).

3.5 Phenotypic detection of efflux pump - mediated drug resistance

3.5.1 Ethidium bromide (EtBr)-agar cartwheel method (screening method): Bacterial isolates were grown in 5.0 ml of LB medium at 37ºC with agitation (220 rpm) until they reached an optical density (OD) of 0.6 at 600 nm. Tryptic soy agar (HiMedia, Mumbai, India) plates containing EtBr concentrations ranging from 0 to 2.5 mg/l were prepared on the same day of the experiment and protected from light. The plates were divided into sectors by radial lines. Cultures were then swabbed onto EtBr agar plates starting from the center of the plate towards the edges and incubated at 37°C for 16 h in dark. The cultures were placed on an UV-transilluminator and photographed using a gel documentation system (AlphaImager 2200, USA). The minimum concentration of EtBr that produced fluorescence of the bacterial mass was recorded, taking corresponding MTCC strains as negative controls (Martins et al., 2010).

3.5.2 Efflux pump inhibitor (EPI)-based microplate assay (confirmatory method): MDR isolates were grown in LB medium until they reached an OD of 0.6 at 545 nm. One ml each of Mueller-Hinton broth was added into 24-well microtiter

plate which also included control wells. Antibiotic discs to be tested were distributed into the wells of the plate and incubated for 1 h at 37ºC. Following the incubation, the efflux pump inhibitor, phenylalanine-arginine beta-naphthylamide (PAN) [Sigma Aldrich Chemicals. Pvt. Ltd.] was dispensed at a concentration of 20 mg/l to the corresponding wells of the microplate. Bacterial suspension (0.1 ml) was inoculated into all the wells and the plates were incubated at 37ºC for 16-18 h. The determination of the effect of PAN was made by comparing the growth of bacterium in the well containing a given antibiotic disc with that of the corresponding well containing the antibiotic disc plus PAN. The contents of the wells with no growth or poorer growth along with the controls were then plated on MHA plates to determine the number of colony forming units (CFU) [Martins et al., 2010].

3.6 Detection of ESBL, MBL and AmpC beta-lactamase production

AmpC-producing phenotypes were detected by AmpC disc test as described by Black et al., 2005. A lawn of *E. coli* MTCC- 41 was made on MHA plate and a cefoxitin (30µg) disc was placed. A sterile disc was rehydrated with 20 µl of sterile saline and inoculated with several colonies of the test organism and placed adjacent to cefoxitin disc. Flattening or indentation of the cefoxitin zone of inhibition in the vicinity of the test organism disc was taken as positive and the undistorted zone was considered as negative.

ESBL status was phenotypically established by combined disc diffusion method using ceftazidime (30µg) disc alone and in combination with clavulanic acid (CAC, 30/10µg discs). The test organism was considered to produce ESBL if the zone of inhibition of CAC disc was ≥ 5 mm in comparison to that produced by ceftazidime disc alone (Easwaran et al., 2016).

Phenotypic MBL production was detected by imipenem-EDTA (IPM-EDTA) combined disc method as described by Yong et al., 2002. Two 10 µg imipenem (IPM) discs were placed on the plate inoculated with the test organism and 10 µl of 0.5 M EDTA solution was added to one of them to obtain a concentration of 750 µg. A zone of inhibition ≥ 7 mm around imipenem with

EDTA disc, when compared to that of imipenem disc alone, indicated MBL production.

3.7 Isolation of genomic DNA

Genomic DNA was isolated from individual bacterial cultures essentially as described by Ausubel et al., 1995. Briefly, 1.5 ml of each bacterial culture at the mid to late-log phase was spun in a microcentrifuge until a compact pellet was formed. The supernatant was discarded and pellet was resuspended in 567µl TE buffer. To this, 30µl of 10% SDS and 3µl of 20mg/ml proteinase K was added, mixed and incubated at 37ºC for 1 h following which 100µl of 5 molar NaCl was added to the solution and mixed thoroughly. Following the addition of 80µl of CTAB/NaCl solution, the mixture was shaken and incubated for 10 min at 65ºC. Equal volume of CIA was added to the solution, mixed and microcentrifuged for 4-5 min. The supernatant was then transferred to a fresh tube and extracted once with phenol-CIA. To the supernatant, 0.6 volume of isopropanol was added and inverted gently to precipitate out the DNA. The DNA pellet was washed with 70% ethanol and the semi-dried pellet was redissolved in 100 µl of TE buffer.

3.8 Isolation of total RNA and synthesis of complementary DNA (cDNA)

Bacterial cultures were grown to mid-exponential phase (OD_{600} = 1.5–2.0) in LB medium and spun briefly to pellet the cells. The pellet was then homogenized thoroughly in 1 ml of TRI reagent® (Sigma Aldrich Chemicals. Pvt. Ltd) and allowed to stand for 5 min at room temperature. To the homogenate 0.2 ml of chloroform was added, mixed vigorously for 15 s and incubated at room temperature for 15 min. The solution was then centrifuged at 12000*g* for 5 min at 4ºC. The supernatant was transferred to fresh tube and RNA was precipitated by mixing with 0.5 ml isopropanol. Following a 5-10 min incubation at room temperature the RNA pellet was recovered by centrifugation at 12000*g* for 10 min at 4ºC. The pellet was then washed with 75% ethanol and the semi-dried pellet was dissolved in sterile double distilled water for subsequent analysis. The residual DNA was removed by adding DNase I (Promega, Madison) according to the manufacturer's instructions.

For cDNA synthesis, a 5 µl reaction containing 0.5 µl of a random hexamer (100 ng/ µl) and 1 µl of 0.5 µg/ µl RNA was incubated at 65ºC for 10 min and placed on ice. To this, added 1 µl of 10mM dNTP mix, 2 µl of 10x M-MLV reverse transcriptase buffer, 1 µl of M-MLV reverse transcriptase, 0.5 µl of RNasin, 0.5 µl DTT (20 mM) and made upto 10 µl with sterile nuclease-free water. The reaction mixture was incubated at 37ºC for 1 h and then heated to 95ºC for 10 min. The reaction was stored at -20ºC until use.

3.9 Spectrophotometric quantification of DNA and RNA

The concentration of DNA/RNA was quantified spectrophotometrically by taking absorbance at 260 nm (an OD value of 1 at 260 nm was taken as equal to 50 µg double stranded DNA or 38 µg single stranded RNA). The purity of nucleic acid was determined by taking the ratio of 260/280 nm absorbance.

3.10 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out in 0.5x TBE as the electrode buffer. Agarose was melted in 1x TBE to cast gels at concentrations ranging from 0.8 to 1.5% (*w/v*). DNA samples were mixed with 6x gel-loading dye and loaded into the wells along with appropriate molecular weight DNA size markers. Electrophoresis was carried out at 100 volt. For visualization the gel was stained with ethidium bromide at a final concentration of 0.25 µg/ml followed by and transferred to the UV- transilluminator for photography (Sambrook et al., 1989).

3.11 Random Amplified Polymorphic DNA (RAPD)

Initial screening of DNA samples was carried out with a set of 25 different decameric oligonucleotide primers - RBa-D (Bangalore Genei, Pvt. Ltd.) to check the reproducibility of the fingerprints. Of them, RBa-D5 primer with the sequence 5'AGGGGCGGCA3' (GenBank accession. no. AM911680) produced different fingerprints for each isolate of *P. aeruginosa* which were consistently reproducible. RAPD reactions were performed according to manufacturer's instructions. Conditions for amplification were as follows: Initial denaturation at 94ºC for 5 min, followed by 8 cycles of 94ºC for 45 s, 35ºC for 1 min, and 72ºC for 1.5 min, 30

cycles of 94ºC for 45 s, 38ºC for 1 min, and 72ºC for 1 min and a final extension step at 72ºC for 10 min. Images of DNA banding patterns obtained after agarose gel electrophoresis were analyzed using the PyElph software (Pavel and Vasile, 2012) to prepare dendrograms using unweighed pair-group method arithmetic mean (UPGMA) method.

3.12 Quantification of gene expressions related to efflux pumps and porin in *P. aeruginosa*

The cDNAs were subjected to semiquantitative/quantitative reverse transcription (RT)-PCR using primers (Eurofins Genomics India Pvt. Ltd., Bangalore) described previously (Table 3.1) under the thermal cycling conditions accomplished after 5 min activation and denaturation step at 95ºC, followed by 40 cycles of 10 s at 98ºC, 30 s at 60ºC and 45 s at 72ºC for specific genes such as *rpsL*, *mexB* , *mexY*, *mexD*, *mexF* and *oprD*.

3.12.1 Semi-quantitative RT-PCR: The RT-PCR reactions were performed using a minicycler (MJ Research, USA) in a reaction volume of 25 µl containing 12.5µl of 2x Emerald GT master mix (TaKaRa Inc., Japan), 0.25 µM of each primer and 0.5 µl of cDNA. Amplification products were analysed on 1% (*w/v*) agarose gels to detect the presence of the expected amplicons and compare the band intensities with those from the reference strain *P. aeruginosa* MTCC- PAO.

3.12.2 Quantitative RT-PCR: The transcript levels of individual mRNA types were determined using SYBR Green PCR Master Mix (TaKaRa Inc., Japan) in an Illumina Eco^{TM} Real-Time PCR system. The relative gene expressions were evaluated using the CT method (Pfaffl, 2001) taking the constitutively expressed *rpsL* gene transcript, encoding ribosomal protein RpsL as representative of housekeeping function. *P. aeruginosa* MTCC-PAO was used as a reference for normalization of relative mRNA levels. The assays were performed in triplicate along with appropriate controls such as reactions containing RNA without reverse transcriptase.

Table 3.1. Details of gene-specific RT- PCR primers

Genes	Primers	Primer sequences (5' – 3')	Reference
rpsL	F	GCAAGCGCATGGTCGACAAGA	(Dumas et al., 2006)
	R	CGCTGTGCTCTTGCAGGTTGTGA	
mexB	F	GTGTTCGGCTCGCAGTACTC	(Yoneda et al., 2005)
	R	AACCGTCGGGATTGACCTTG	
mexY	F	CCGCTACAACGGCTATCCCT	(Yoneda et al., 2005)
	R	AGCGGGATCGACCAGCTTTC	
mexD	F	CGAGCGCTATTCGCTGC	(Xavier et al., 2010)
	R	GGCAGTTGCACGTCGA	
mexF	F	CGCCTGGTCACCGAGGAAGAGT	(Xavier et al., 2010)
	R	TAGTCCATGGCTTGCGGGAAGC	
oprD	F	TCCGCAGGTAGCACTCAGTTC	(Savli et al., 2003)
	R	AAGCCGGATTCATAGGTGGTG	

3.13 Amplification of genomic DNA for sequencing of efflux pumps and porin related genes in *P. aeruginosa*

The PCR reactions were performed essentially as already described in section 3.12.1 but with 100 ng of genomic DNA as template. The regulatory genes associated with different RND pumps taken up for amplification were *mexR, nalC* and *nalD* for MexAB-OprM; *mexZ* for MexXY; *nfxB* for MexCD-OprJ; *mexT* for MexEF-OprN. Additionally, the *oprD* gene encoding porin channel protein was also amplified. Primers used for DNA amplifications and sequencing (Eurofins Genomics India Pvt. Ltd., Bangalore) are listed in Table 3.2 and thermal cycling conditions are given in Table 3.3. PCR products were cloned using CloneJET PCR cloning kit in accordance with the manufacturer's instructions and then transformed into *E.coli* DH5α strain. Recombinant plasmids were isolated using alkaline lysis method and sequenced at commercial facilities (Eurofins Genomics India Pvt. Ltd., Bangalore; SciGenom Labs Pvt. Ltd., Kochi). The amplicon sequences were analyzed with BLAST tool of NCBI (www.ncbi.nlm.nih.gov) by comparison with sequences of the reference strain, *P. aeruginosa* PAO1 retrieved from the GenBank

database. The translation of the nucleic acid sequences into amino acids was performed using ExPasy Bioinformatics Resource Portal (http://web.expasy.org/translate) and ORF Finder (www.ncbi.nlm.nih.gov/orffinder/). The resulting protein sequences were aligned and analysed using Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo/).

3.13.1 Preparation of *E.coli* DH5α competent cells and their transformation

A single *E.coli* DH5α bacterial colony was inoculated into 5.0 ml of LB medium and grown in an incubator shaker at 37ºC overnight. A subculture of the *E.coli* DH5α (1/100th volume) in 50 ml of growth medium was grown for 3 h at 37^{o}C with vigorous shaking. Competent cells were then prepared using 100 mM $CaCl_2$ solution and aliquoted at 100 µl per eppendorf tube (Sambrook and Russell, 2001). For transformation 2.0 µl each of the ligated plasmids, carrying the gene-specific amplicons as inserts, was added to 100 µl of the competent cells and kept on ice for 30 min. Then a heat shock at 42ºC was given for exactly 90s followed by a quick chill for 2.0 min. To this 900 µl of prewarmed (37ºC) LB medium was added and incubated at 37ºC for a period of 45 min - 1 h.

3.13.2 Isolation of recombinant plasmid DNA by alkaline lysis method

1.5 ml of each of the transformed clones taken in microfuge tubes were pelleted after 10 min centrifugation. The pellets were then resuspended in 0.5 ml of STE (Sodium Chloride Tris EDTA) and centrifuged for 10 min and supernatant was removed. The pellet was kept on ice, to which 100 µl of ice-cold Solution I (GTE-Glucose Tris EDTA) was added and mixed thoroughly. Then 200 µl of freshly prepared solution II (0.2 N sodium hydroxide in 1.0% *v/v* SDS) was added, mixed gently and incubated the tube on ice for 10-15 min. After incubation, 150 µl of solution III (5 M Potassium acetate in glacial acetic acid) was added, mixed and kept on ice for 5 min. The tube was then centrifuged at 10000*g* and the supernatant was carefully transferred to new tubes. Equal volume of phenol-CIA was added to the supernatant, mixed and centrifuged at 10000*g* for 5 min at 4ºC. This step was repeated till the aqueous phase became clear. To the supernatant, equal volume of CIA was added, mixed and recentrifuged at 10000*g* for 5 min at 4ºC. The plasmid DNA was then precipitated by the addition of two volumes of ice-cold distilled

ethanol. Following a 70% ethanol wash the semi-dried pellet was dissolved in TE buffer (pH 8.0) and stored at -20ºC (Sambrook and Russell, 2001).

Table 3.2. Details of gene-specific primers used for amplification and sequencing of PCR amplified DNA

Genes	Primers	Primer sequences (5' – 3')	Reference
mexR	F	TGTTCTTAAATATCCTCAAGCGG	(Quale et al., 2006)
	R	GTTGCATAGCGTTGTCCTCA	
nalC	F	TCAACCCTAACGAGAAACGCT	(Quale et al., 2006)
	R	TCCACCTCACCGAACTGC	
nalD	F	GCGGCTAAAATCGGTACACT	(Sobel et al., 2005)
	R	ACGTCCAGGTGGATCTTGG	
mexZ	F	ATTGGATGTGCATGGGTG	(Sobel et al., 2003)
	R	TGGAGATCGAAGGCAGC	
nfxB	F	ACGCGAGGCCAGTTTTCT	(Vaez et al., 2014)
	R	ACTGATCTTCCCGAGTGTCG	
mexT	F	AAAACCACCCGTCGTTATTG	(Quale et al., 2006)
	R	CAGTTCGTCGGTGTAGCTGA	
oprD	F	CTACGCAGATGCGACATGC	(Wolter et al., 2004)
	R	CCTTTATAGGCGCGTTGCC	

Table 3.3. Gene-specific thermal cycling conditions

Gene	Cycling conditions			
	Denaturation	**Annealing**	**Extension**	**No.of cycles**
mexR	95ºC for 30 sec	56ºC for 1.30 min	72ºC for 1 min	35
nalC	95ºC for 1 min	56ºC for 1 min	72ºC for 1 min	30
nalD	94ºC for 40 sec	61ºC for 1 min	72ºC for 1 min	30
mexZ	94ºC for 1 min	55ºC for 1 min	72ºC for 1 min	30
nfxB	95ºC for 1min	60ºC for 45 sec	72ºC for 1min	35
mexT	95ºC for 30 sec	56ºC for 1.30 min	72ºC for 1 min	35
oprD	95ºC for 30 sec	56ºC for 30 sec	72ºC for 2 min	25

3.14 Phylogenetic Analysis

The DNA sequences of efflux pump and porin related genes in *P. aeruginosa* obtained in the present study were compared with similar sequences of *P. aeruginosa* available in the NCBI database. Minimal 'E' values, maximum query coverage, including geographical location of the source organisms, were the factors considered for sequence selection. All sequences were aligned using default configuration of multiple sequence comparison by clustalW embedded in MEGA6 software (http://www.megasoftware.net). Nucleotide sequence evolution with respect to each gene was carried out using the software, jModelTest (Posada, 2008) based on Akaike Information Criterion (AIC). The phylogram was constructed by Bayesian inference using Markov Chain Monte Carlo method (Huelsenbeck and Ronquist, 2001).

3.15 Molecular modelling of OprD protein

The 3D molecular model of the OprD porin channel protein was built by homology modelling approach employing Modeller 9.18 (2018 Version) software. The chain-A of OprD from *P.aeruginosa*, Protein Data Bank (PDB): 4FOZ was identified as the best template for comparative modelling. Stereochemical analysis of the structure was evaluated using Ramachandran plot generated by PROCHECK (Laskowski et al., 1993). Energy minimization of the modelled protein was refined and validated by three different molecular dynamic force fields named ANOLEA (Melo et al., 1997), QMEAN (Benkert et al., 2009), GROMOS (Christen et al., 2005) and secondary structure by DSSP(Kabsch and Sander, 1983). Energy minimized modelled structure was visualized by PyMOL software (Seeliger and de Groot, 2010).

3.16 Restriction digestion

Restriction digestion reactions were set up in a total reaction volume of 10 µl containing 5µl of the initial 25 µl *oprD* gene amplification reaction. Three such reaction sets were separately restricted (1.0 unit of enzyme / µg DNA) with each of *Hin*c II, *Pst* I and *Pvu* II restriction enzymes in the corrosponding 1x restriction

buffers (Bangalore Genei, Pvt. Ltd.). The reaction mixtures were then incubated at the prescribed temperature (37°C) for 1 h. The restriction digests were electrophoresed on 1% agarose gels followed by visualization and photography as described in section 3.10. For detection of the putative restriction sites within the PCR amplicons obtained from the *oprD* gene, NEB cutter V2.0 (Vincze et al., 2003) was utilized.

3.17 Statistical analysis

Categorical variables were compared by χ^2 test using SPSS software version 20.0. A significant difference between two proportions was checked by *Z*-test using MedCalc Statistical Software Version 17.9.7. A two-tailed P value of <0.01 or <0.05 was considered significant.

RESULTS AND DISCUSSION

4.1 Identification of bacterial isolates

A total of 144 MDR gram-negative bacterial isolates were collected from various clinical laboratories in Kerala. These bacterial isolates were received in pure line form following preliminary identifications already carried out in the respective laboratories. The identity of each isolate was then reconfirmed by gram staining, motility testing and biochemical reactions (Table 4.1). The tested isolates belonged to 4 genera comprising of *Klebsiella* spp. (n=50), *E. coli* (n=46), *P. aeruginosa* (n=33) and *Acinetobacter* spp. (n=15). Fig 4.1 shows the overall percentage distribution of bacterial isolates used in the present study.

Table 4.1. Bacterial identification - Gram staining and biochemical reactions

Isolates	Gram reaction	Indole	MR	VP	Citrate utilization	Mannitol fermentation	Motility	Oxidase
E.coli	-	+	+	-	-	+	+	-
Klebsiella spp.	-	-	-	+	+	+	-	-
P. aeruginosa	-	-	-	-	+	-	+	+
Acinetobacter spp.	-	-	-	-	+	-	-	-

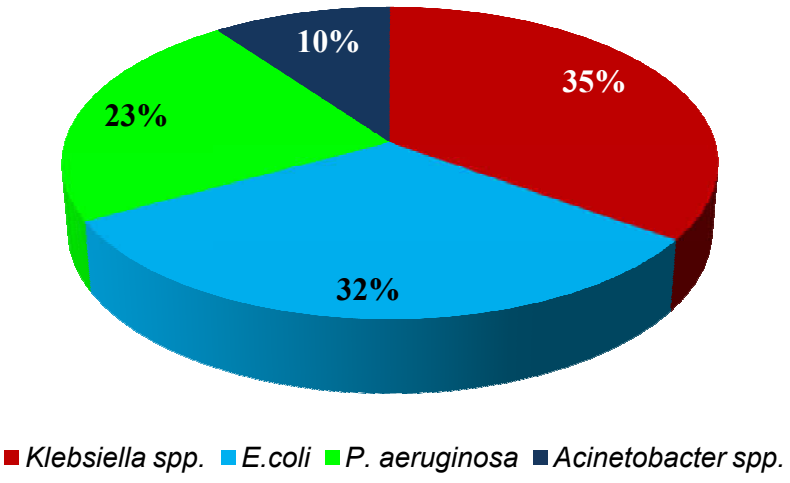


Fig. 4.1. Overall percentage distribution of MDR gram-negative clinical isolates.

4.2 Antibiotic sensitivity

Antibiotic sensitivity of the 144 bacterial isolates were detected by Kirby-Bauer disc diffusion method based on the recommended guidelines (CLSI, 2012). *E. coli* and *Klebsiella* spp. were tested with 14 antibiotic discs such as amikacin-30µg (AK), ampicillin-10 µg (AMP), aztreonam-30µg (AT), cefotaxime-30µg (CTX), ceftazidime- 30µg (CAZ), cefepime-30µg (CPM), chloramphenicol-30µg (C), ciprofloxacin-5µg (CIP), gentamicin-10µg (GEN), meropenem-10µg (MRP), nalidixic acid-30µg (NA), ofloxacin-5µg (OF), piperacillin/tazobactam-100/10µg (PIT) and tetracycline-30µg (TE). For *P. aeruginosa* and *Acinetobacter* spp., antibiotic sensitivity assay was performed with 16 antibiotic discs including colistin-10µg (CL) and polymyxin B-300 units (PB) in addition to the above mentioned antibiotics.

The antibiotic resistance profiles of isolates are given in Figs. 4.2 and 4.3. All isolates were found to be ampicillin-resistant. Of these, *E. coli* isolates also exhibited complete resistance to 3rd generation cephalosporins (CTX, CAZ) and to CIP, a quinolone antibiotic. Prevalence of resistance against antibiotic C was, however, observed to be comparatively lowest in *Klebsiella* spp. and *E. coli* at 52% and 22% respectively. Incidentally, all *P. aeruginosa* isolates showed resistance against the quinolone, NA with only 48% of them being PB-resistant. The 15 *Acinetobacter* spp. were also found to display resistance against monobactam (AT), CTX, 4th generation cephalosporin (CPM), CIP, beta-lactam/beta-lactam inhibitor combination (PIT), TE and CL.

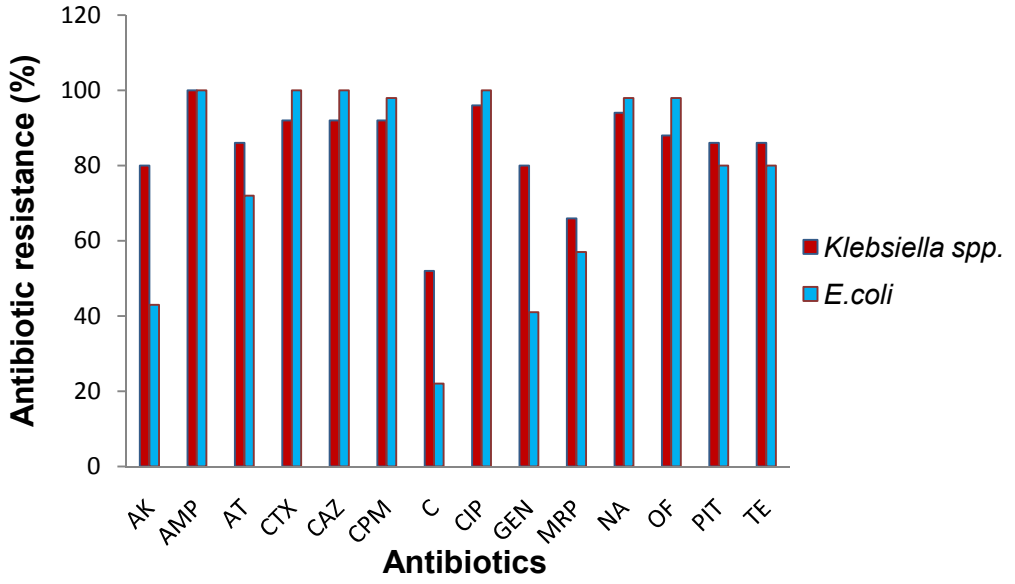


Fig. 4.2. Overall antibiotic resistance profile of E. coli and Klebsiella spp. against 14 antibiotics used in the study.

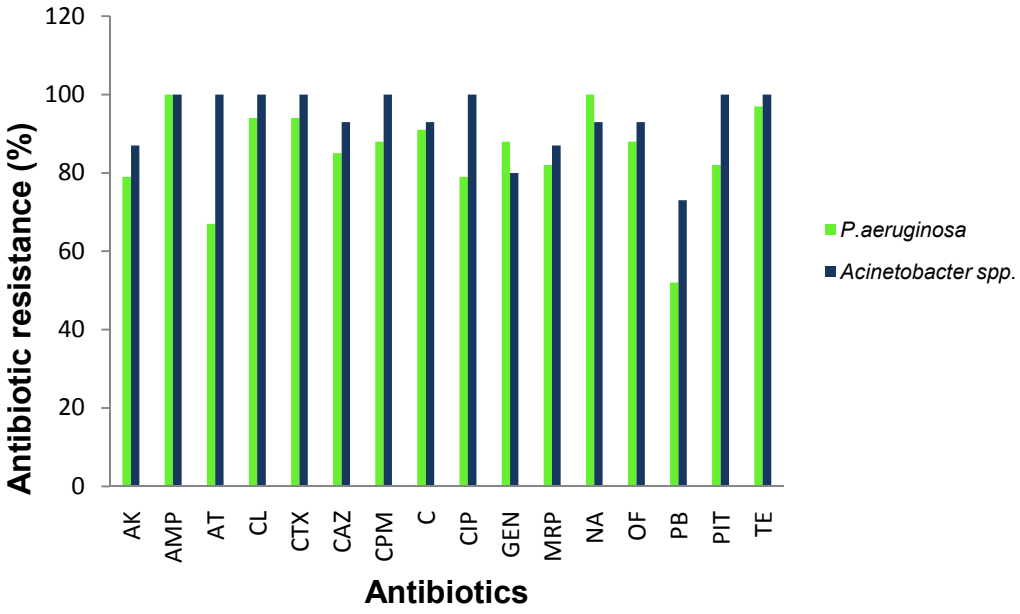


Fig. 4.3. Overall antibiotic resistance profile of P. aeruginosa and Acinetobacter spp. against 16 antibiotics used in the study.

4.2.1 Multiple Antibiotic Resistance (MAR) index

MAR indexing has been shown to be a cost effective and valid method for tracking bacterial source and spread of resistance in a given population. The MAR index value was determined for each isolate by dividing the number of antibiotics to which the isolate was resistant with the total number of antibiotics evaluated for susceptibility assay (Krumperman, 1983). MAR index values greater than 0.2 indicated that bacteria originated from an environment where several antibiotics were used (Hora and Ali, 2012; Sandhu et al., 2016) and the index value of 1.0 denoted resistance to all the antibiotics tested. The MAR index of the isolates *Klebsiella* spp., *E. coli, P. aeruginosa* and *Acinetobacter* spp. ranged from 0.29 to 1.0, 0.36 to 1.0, 0.50 to 1.0 and 0.56 to 1.0 respectively (Figs. 4.4 and 4.5). A MAR index value of 1.0 was observed in 37% (n=17) of *Klebsiella* spp., 9% (n=4) of *E. coli,* 24% (n=8) of *P. aeruginosa* and 53% (n=8) of *Acinetobacter* spp. Overall, the majority of the isolates of *Klebsiella* spp. (n=17), *E. coli* (n=14), *P. aeruginosa* (n=10) and *Acinetobacter* spp. (n=8) displayed a MAR index of 1.0, 0.79, 0.94 and 1.0 respectively.

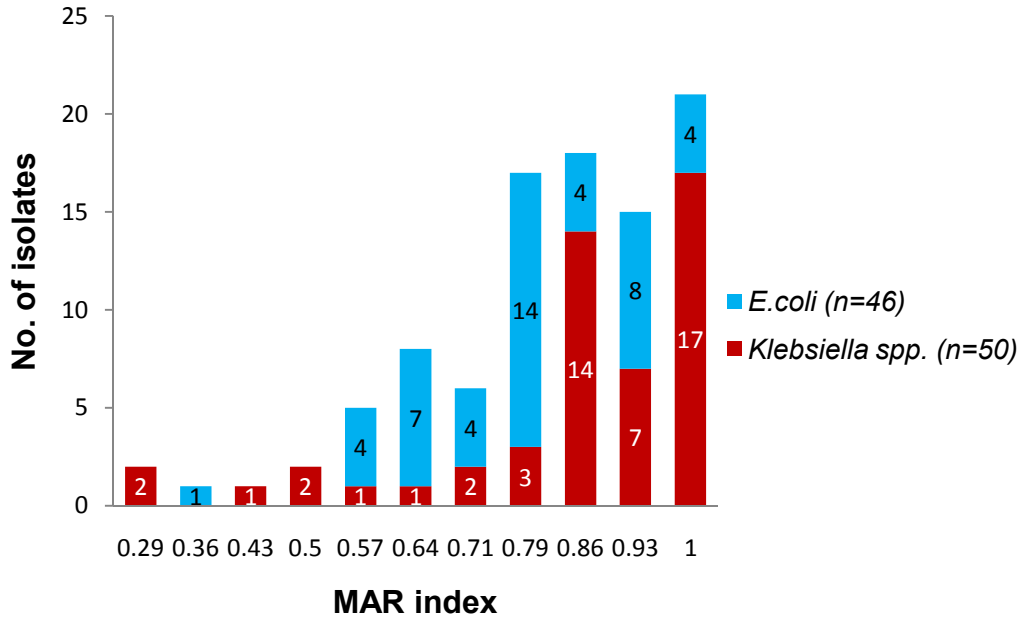


Fig. 4.4. MAR index profiling of Klebsiella spp. and E. coli.

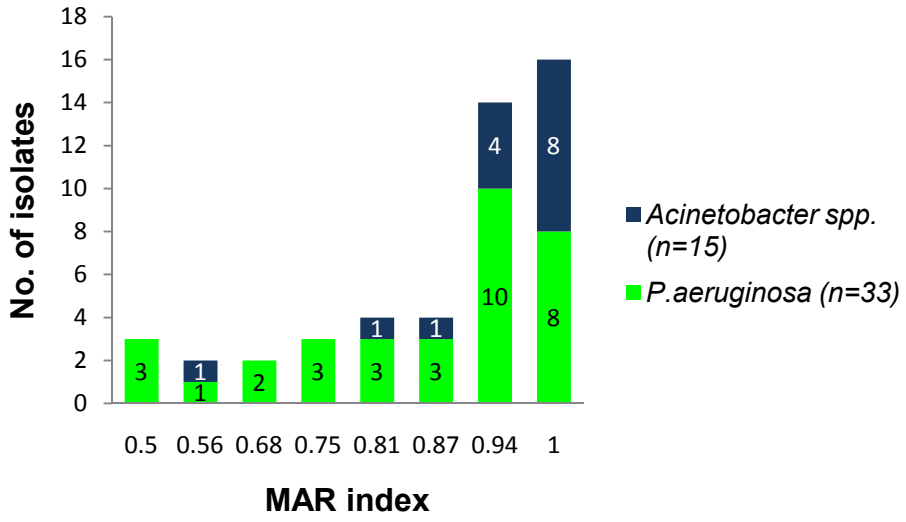


Fig. 4.5. MAR index profiling of P.aeruginosa and Acinetobacter spp.

4.3 Analysis of efflux pump-mediated drug-resistance by phenotypic methods

Efflux pump-mediated drug-resistance was detected in MDR isolates (n=144) by two simple phenotypic methods, namely, i) ethidium bromide (EtBr)-agar cartwheel method and ii) efflux pump inhibitor (EPI)-based microplate assay.

4.3.1 EtBr-agar cartwheel method

A preliminary screening of the bacterial cultures resulted in detection of efflux pump activity in 31 isolates ($P<0.01$) as evidenced by fluorescence of EtBr (Fig. 4.6, Table 4.2). The minimum detectable limit of fluorescence as obtained in the presence of 0.5mg/l of EtBr in the MTCC strain (negative control) served as an indication of the absence of efflux pump activity. Based on this criterion, clinical isolates without efflux pump activity were found to be represented in all 4 genera. Fluorescence, detectable in the presence of EtBr, at or above1.0 mg/l concentration was considered as positive for efflux pump activity (Martins et al., 2010 and 2011). At 1.0 mg/l EtBr concentration, a few isolates from all 3 genera, namely, *E. coli*, *Acinetobacter* spp. and *Klebsiella* spp., were found to fluoresce. Interestingly, *P. aeruginosa* isolates showed much higher efflux pump activity in comparison to isolates from other genera. Out of 33 *P. aeruginosa* isolates, as many as 17 effectively effluxed the fluorochrome dye at concentrations which were many folds higher reaching upto 2.5mg/l (Table 4.2).

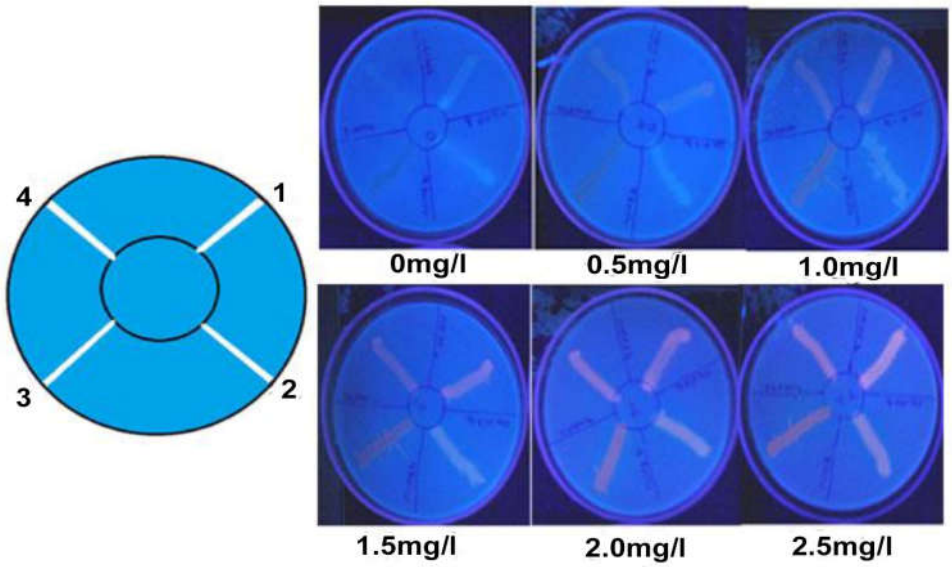


Fig. 4.6. EtBr-agar cartwheel method. *Tryptic soy agar petri-plates containing varying concentrations of EtBr were swabbed with P. aeruginosa isolates. A schematic representation of a Petri-plate on the left of the figure denotes the position of bacterial isolates swabbed outwards from the centre - (1) P. aeruginosa MTCC 2453 (negative control),(2) P. aeruginosa clinical isolate positive for efflux pump activity-based fluorescence detectable in the presence of EtBr at 1.0 mg/l, (3) and (4) P. aeruginosa clinical isolates without efflux pump activity.*

Table 4.2. Determination of efflux-pump activity in the presence of varying concentrations of the fluorochrome – ethidium bromide

No. of bacterial isolates	Concentration of EtBr at which bacteria started to fluoresce	Efflux pump activity
***Klebsiella* spp. (n=50)**		
49	<0.5mg/l	-
1	1mg/l	+
***E.coli* (n=46)**		
34	<0.5mg/l	-
12	1mg/l	+
***P. aeruginosa* (n=33)**		
16	<0.5mg/l	-
9	1mg/l	+
7	1.5mg/l	+
1	2.5mg/l	+
***Acinetobacter* spp. (n=15)**		
14	<0.5mg/l	-
1	1mg/l	+

'+ and –' represent presence and absence of efflux pump activity respectively

Efflux systems play a key mechanistic role in the development of drug resistance in gram-negative bacteria. On the basis of literature scan, incidentally, this is the first report from the State of Kerala on efflux pump-mediated drug-resistance among MDR gram-negative bacteria. The EtBr-agar cartwheel screening method revealed efflux activity in 21.5% of the isolates used in the study. It is relevant to point out the likelihood of recording false positives during such type of screening, since bacterial permeability to EtBr may also be influenced by down-regulation of porins leading to decreased influx of EtBr. This view is supported by the observation that porin mutants show reduced entry of EtBr. In other words, this implies that these channels might be involved in the passive influx of the molecule (Rodrigues et al., 2011).

4.3.2 EPI -based microplate assay for confirming efflux pump activity

The 31 isolates identified to possess efflux pump activity by the cartwheel method were subjected further to an EPI-based microplate assay employing selected antibiotics against which resistance was observed (Fig. 4.7). In the presence of the efflux pump inhibitor, PAN, some isolates displaying efflux pump activity completely reverted to a phenotype sensitive to the antibiotic(s) concerned (denoted as 'reversal' in Table 4.3). In other words, resistance to specific antibiotics in these isolates was solely due to efflux pumping activity. Isolates with reduced growth compared to controls were indicative of only a partial contribution of efflux pump activity towards antibiotic resistance (denoted as 'reduction' in Table 4.3). Based on the above mentioned criteria, only 19 isolates tested positive for efflux pump activity. Of these, 7 isolates displayed efflux pump activity against more than one antibiotic (Table 4.3). Further, efflux pump-mediated drug-resistance was found to be most prevalent in *P. aeruginosa* (27.3%, n=9), followed by that in *E. coli* (17.4%, n=8), *Acinetobacter* spp. (6.7%, n=1) and *Klebsiella* spp. (2%, n=1).

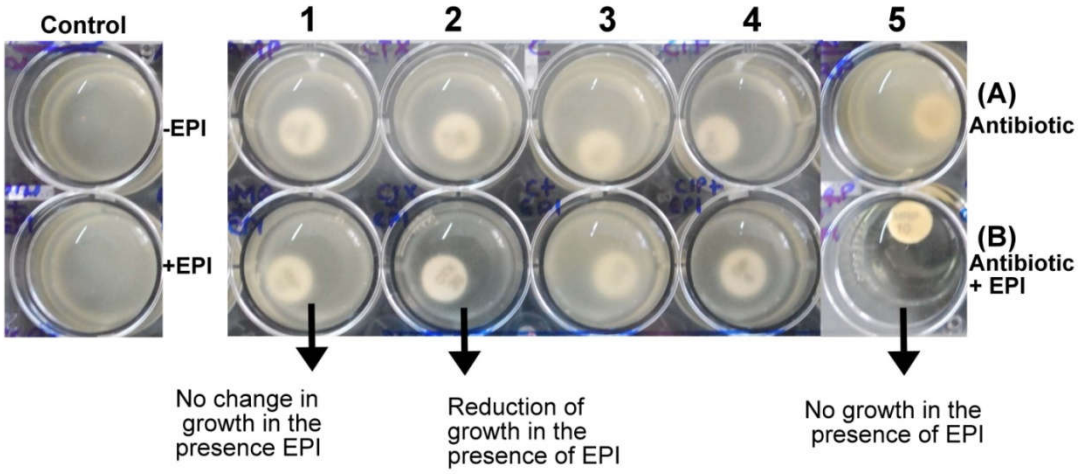


Fig. 4.7. Efflux pump inhibitor (EPI)-based microplate assay*. Control-wells containing bacterial culture with (+EPI) or without (-EPI) inhibitor; columns (1-5) contain antibiotic discs such as ampicillin, cefotaxime, chloramphenicol, ciprofloxacin and meropenem respectively; first row (A) contains only antibiotic discs; second row (B)contains EPI along with antibiotic discs.*

In respect of the results mentioned above, it is to be noted that according to the standard Kirby Bauer chart, the criterion used to define resistance includes consideration of diameter of zone of inhibition ranging from zero to a few millimeters as specified for the respective antibiotic. In the present study, unambiguous and reproducible results were obtained only when bacterial isolates exhibiting zero zone of inhibition against the respective antibiotics were used.

Table 4.3. Effect of efflux pump inhibitor (phenylalanyl arginyl-beta-naphthylamide) on antibiotic resistance

Bacterial isolates	**Antibiotics**	**CFU ($x10^5$)**		**EPI Activity**
		With antibiotic alone	**With antibiotic + EPI**	
Klebsiella spp.				
Kb11	C	204000	3200	Reduction
E. coli				
Ec4	CTX	53500	1100	Reduction

Ec12	AK	7700	0.001	Reduction
	TE	20	0.001	Reduction
Ec13	AK	13400	0	Reversal
	PIT	148	0	Reversal
	TE	36	0	Reversal
Ec19	NA	4500	73	Reduction
Ec25	C	85	9.1	Reduction
	TE	6000	0	Reversal
Ec26	TE	1600	0	Reversal
Ec30	TE	2.33	0	Reversal
Ec33	NA	40000	12	Reduction
P. aeruginosa				
Pa1	C	3400	0	Reversal
Pa2	CTX	24000000	92	Reduction
	MRP	11500	0	Reversal
Pa5	CTX	30800000	23800	Reduction
	C	642000	0	Reversal
	NA	1382000	2	Reduction
	TE	5820000	0	Reversal
Pa6	CTX	1970000	0	Reversal
	C	15300	0	Reversal
Pa7	C	450000	0	Reversal
Pa13	MRP	4100000	0.002	Reduction
	OF	387000000	74000	Reduction
	TE	37200	0.006	Reduction
Pa16	TE	151000000	6.5	Reduction
Pa25	C	37300	0.002	Reduction
Pa29	TE	262000	0	Reversal
***Acinetobacter* spp.**				
Ac6	TE	23500	1.09	Reduction

AK- amikacin, CTX- cefotaxime, C- chloramphenicol, MRP- meropenem, NA- nalidixic Acid, OF- ofloxacin, PIT- piperacillin/tazobactam, TE- tetracycline, CFU- colony forming units, EPI- efflux pump inhibitor. E. coli and P. aeruginosa isolates were found to efflux more than one antibiotic.

4.4 Phenotypic and molecular investigations for characterization of MDR clinical isolates of *P. aeruginosa*

Phenotypic analysis of efflux pump activity, described in section 4.3.2, revealed a relatively higher incidence of efflux pump based antibiotic resistance mechanism in the genus *P. aeruginosa* compared to isolates belonging to other genera. *P. aeruginosa* is now categorized as a 'superbug' due to the acquisition of multidrug-resistant phenotypes through (i) intrinsic resistance mechanisms such as those mediated by constitutive expression of AmpC beta-lactamase, efflux pumps and porin down-regulation and (ii) acquired resistance caused by mutational changes or acquisition of resistance mechanisms via horizontal gene transfer. Together, these processes contribute toward development of overwhelming resistance against a variety of structurally unrelated antibiotics leading to difficulties in therapy or its failure (Poole, 2011).

All further investigations including phenotypic detection of various beta-lactamases, RAPD analysis, efflux pump and porin-related gene expression/mutation studies were focused on MDR clinical isolates of *P. aeruginosa.* Of the 33 isolates of *P. aeruginosa*, barring 4 which failed to revive during subculturing, the remaining 29 designated as Pa1-Pa29 were subjected to phenotypic and molecular characterizations.

4.4.1 Phenotypic prevalence of AmpC beta-lactamase, ESBL and MBL

The results of antibiotic sensitivity assay presented in section 4.2 revealed that *P. aeruginosa* isolates possessed >80% resistance against cephalosporins and MRP. Several mechanisms such as overexpression of chromosome-mediated AmpC-cephalosporinases, acquisition of transferable beta-lactamase genes, overproduction of efflux pump systems and reduced permeability due to porin downregulation are held responsible for beta-lactam antibiotic resistance (Livermore, 2002). Beta-lactamases encoded by both chromosomal and plasmid genes hydrolyze and destroy broad-spectrum beta-lactam antibiotics and pathogens harbouring them are thus considered a serious health threat due to limited treatment options (Ansari et al., 2016). Effective therapeutic regimens heavily rely on strain-

specific knowledge on the status of different types of beta-lactamase (s) production either singly or in combination since the gene (s) concerned are highly likely to be acquired or can undergo mutations under variations of antibiotic pressure (Cabot et al.,2016; López-Causapé et al., 2017). Hence, in this study phenotypic detection of the enzymes AmpC beta-lactamase, ESBL and MBL production were also ascertained.

AmpC disc test was used for the detection of AmpC beta-lactamase production as described by Black et al. (2005). A cefoxitin disc and a sterile disc inoculated with several colonies of the test organism were placed adjacent to each other on a MHA with a lawn of *E. coli* MTCC 41 and incubated. Flattening or indentation of the cefoxitin zone of inhibition in the vicinity of the test organism disc was interpreted as a 'positive' result whilst the presence of an undistorted zone was considered 'negative'(Fig 4.8-a). Analysis of the results revealed the incidence of *P. aeruginosa* with AmpC-producing phenotypes to be 31% (n=9; Table 4.4). ESBL activity was determined by combined disc diffusion method using CAZ disc alone and in combination with clavulanic acid (CAC). Zone of inhibition around CAC disc was found to increase in diameter (> 5 mm) than that around CAZ in case of *P. aeruginosa* and the incidence in this case was observed to be 13.8% (n=4; Fig 4.8-b and Table 4.4). The presence or overexpression of inducible chromosomal AmpC beta-lactamase in *P. aeruginosa* is likely to interfere with or even hide the detection of ESBLs by phenotypic tests (Laudy et al., 2017). Phenotypic detection of ESBL was also found to be difficult in isolates producing both carbapenemases and ESBLs simultaneously (Ellappan et al., 2018). The MBL enzymes which hydrolyze carbapenems, the major anti-pseudomonal antibiotic, were detected by the imipenem-EDTA combined disc test. MBL-positive isolates which showed a zone of inhibition ≥ 7 mm around imipenem with EDTA disc comparable to that of imipenem (IPM) disc alone amounted to 44.8% (n=13; Fig 4.8-c and Table 4.4). AmpC and MBL co-producers constituted 27.6% (n=8) with only a single isolate co-producing ESBL and MBL. About 41.4 % (n=12) of the isolates were found lacking in the expression of any of the three enzymes tested. Percentage distribution of beta-lactamases is given in Fig. 4.9.

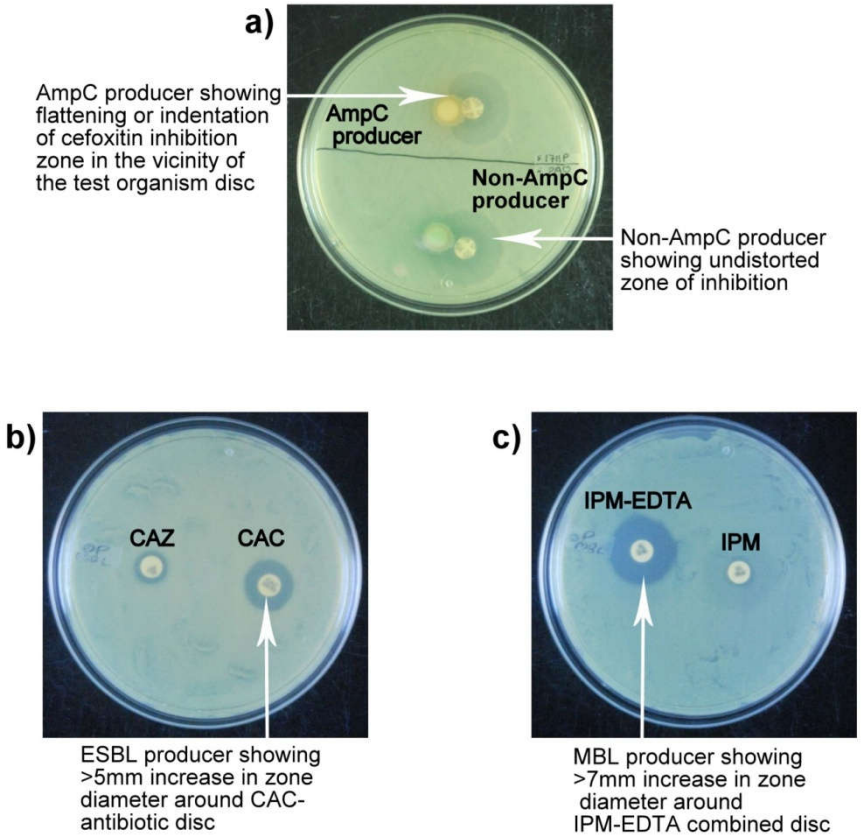


Fig. 4.8. MHA plates showing AmpC, ESBL and MBL activity.

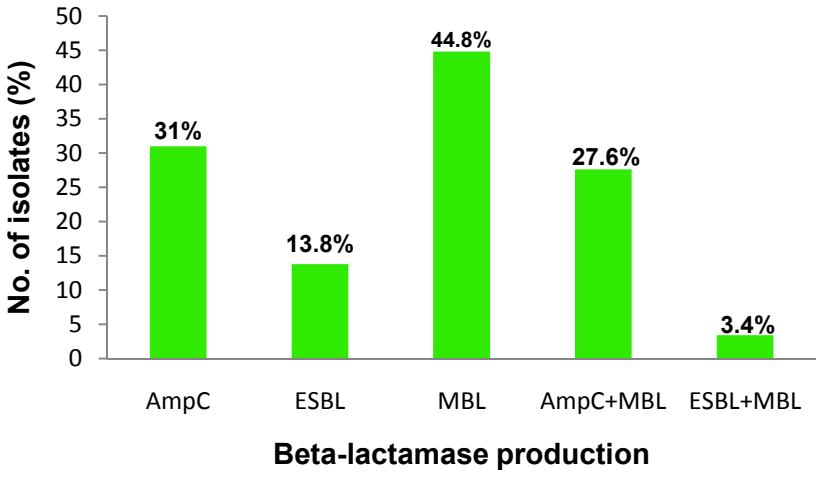


Fig. 4.9. Percentage distribution of various beta-lactamase producers in clinical isolates of P. aeruginosa (P<0.05).

Table 4.4. Beta-lactamase production in *P. aeruginosa* clinical isolates

Isolates	AmpC	ESBL	MBL	Isolates	AmpC	ESBL	MBL
Pa1	–	–	–	Pa16	+	–	+
Pa2	+	–	–	Pa17	–	–	–
Pa3	+	–	+	Pa18	–	–	+
Pa4	–	+	–	Pa19	–	+	+
Pa5	–	–	–	Pa20	–	–	–
Pa6	–	–	–	Pa21	–	–	+
Pa7	+	–	+	Pa22	–	–	–
Pa8	+	–	+	Pa23	–	+	–
Pa9	–	–	–	Pa24	–	–	–
Pa10	–	–	+	Pa25	+	–	+
Pa11	–	–	–	Pa26	–	–	–
Pa12	–	+	–	Pa27	–	–	–
Pa13	+	–	+	Pa28	–	–	–
Pa14	–	–	+	Pa29	+	–	+
Pa15	+	–	+				

ESBL, extended-spectrum beta-lactamase; MBL, metallo-beta-lactamase; '+ and –' represent beta-lactamase and non-beta-lactamase producers, respectively.

4.4.2 RAPD typing and analysis of clonally related isolates

Given the fact that certain DNA sequences, also known as 'hot spots' show differential propensity in accumulating mutations, RAPD typing was carried out in an attempt to gauge genetic diversity amongst the bacterial clinical isolates of MDR *P. aeruginosa* under study (Couce et al., 2016). This technique is relatively faster, inexpensive and technically less demanding with low turnaround time as compared to the other typing methods (Podder et al., 2014). RAPD profiles in this study produced from *P. aeruginosa* isolates employing random decameric primer - RBa-D5 (GenBank accession no. AM911680) generated reproducible and distinctive patterns (Fig. 4.10). These profiles were analyzed using open source Python-based software, PyElph to estimate the genetic similarity by Dice coefficients of correlation and generate a dendrogram by the UPGMA clustering (Pavel and Vasile, 2012). The dendrogram showed two major clusters - *A* and *B*, in which major cluster *A* was found to be subdivided into two sub-clusters *A1* and *A2* (Fig. 4.11). Most of the isolates with efflux pump activity were found to be included in the same sub-cluster *A1*. Of them Pa1, Pa5, Pa7 and Pa13 belonged to a single clade whilst strains such as Pa2 and Pa6 were found grouped into another clade. Majority of the

isolates (Pa15, Pa16, Pa20, Pa23, Pa24 and Pa25) possessing MAR index 1.0 fell under sub-cluster *A2*. Among them similarities were observed in isolates including Pa15, Pa25, Pa20 and Pa23. Non-ESBL producers such as Pa16 and Pa24 were found to align into a single clade.

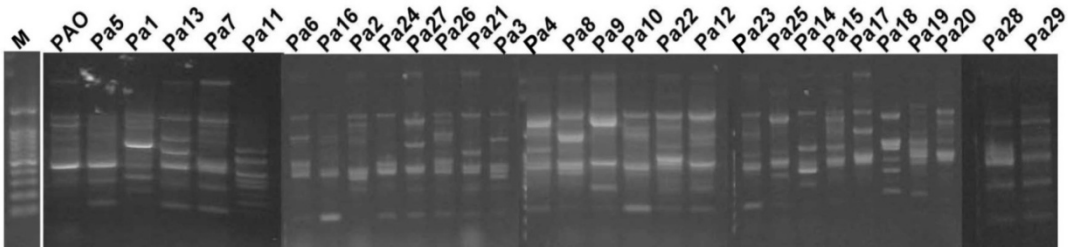


Fig. 4.10. RAPD profiles of reference strain PAO (MTCC) and MDR P. aeruginosa clinical isolates. *Lane denoted M represents 100bp DNA ladder.*

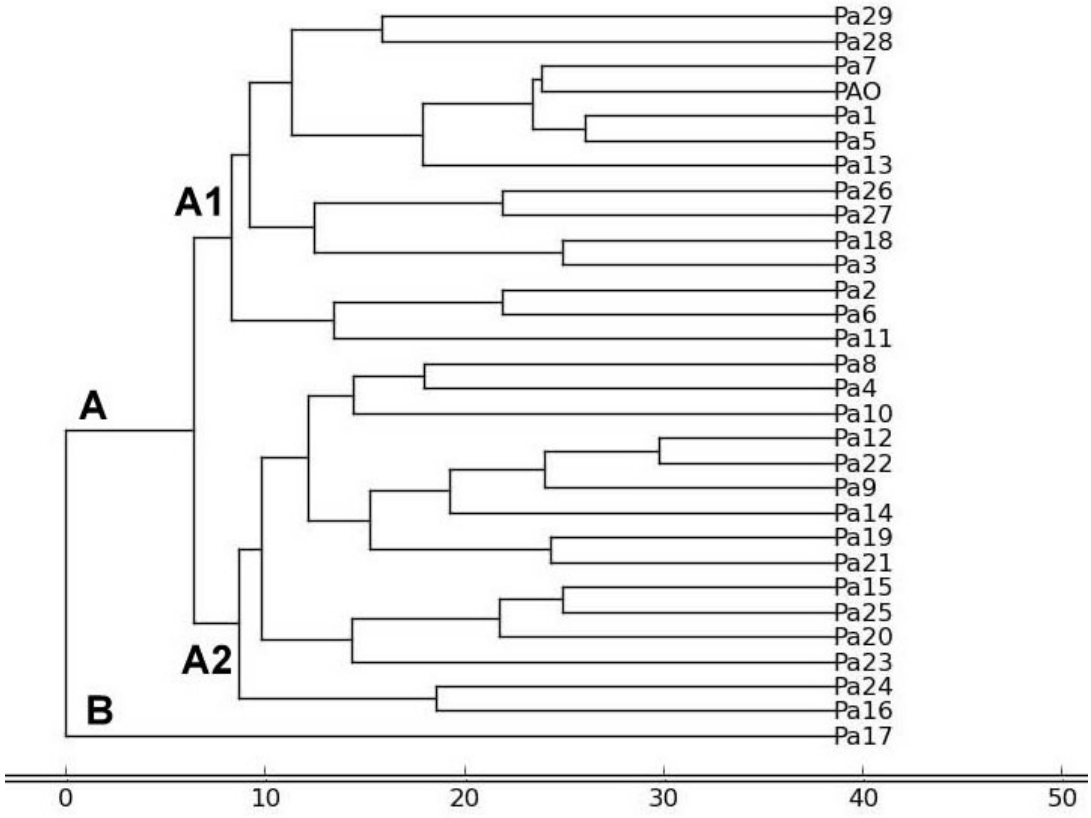


Fig. 4.11. RAPD dendrogram showing clonal relatedness amongst P. aeruginosa.

4.4.3 Expression of efflux pumps, *mexB*, *mexY* , *mexD, mexF* and porin, *oprD* genes

Relative expression levels of transcripts from *mexB*, *mexY* and *mexD* genes were studied by semi-quantitative and real-time RT-PCR technique. For semiquantitative and quantitative assessment, all mRNA transcripts were normalized against the housekeeping gene *rpsL* transcript encoding 30S ribosomal protein S12. For semiquantitative analysis, band intensities of expected amplicons in clinical isolates were compared with those from the reference strain *P. aeruginosa* MTCC-PAO (Fig. 4.12). Expected amplicon sizes with respect to *rpsL*, *mexB*, *mexY* and *mexD* genes are 201, 244, 250 and 165 bp respectively (Yoneda et al., 2005; Dumas et al., 2006; Xavier et al., 2010). However, *mexB* was significantly overexpressed in three (Pa6, Pa13 and Pa16), *mexY* in six (Pa5, Pa6, Pa13, Pa16, Pa25 and Pa29) and *mexD* in three isolates (Pa6, Pa7 and Pa13). These results were correlated with those obtained with quantitative RT-PCR, wherein the target genes were considered overexpressed when their transcriptional levels were at least three folds higher than those of the reference strain (Islam et al. 2004). Gene expression analysis of *P. aeruginosa* revealed that 10.3% (n=3), 20.7% (n=6) and 10.3% (n=3) of isolates displayed increased transcription of *mexB*, *mexY* and *mexD* respectively (Table 4.5), corroborating the results of semi-quantitative RT-PCR. The overexpression ranged from 5.02 - 11.08 folds for *mexB*, 3.61 - 26.17 for *mexY* and 4.08 - 14.42 for *mexD* in comparison with those of PAO. Notably, simultaneous expression of MexY was observed in MexB over-producer Pa16, a co-expression previously reported (Llanes et al., 2004). Two isolates, Pa6 and Pa13, simultaneously expressed *mexB*, *mexY* and *mexD*. The presence of *mexB* transcript in Pa18, Pa28 and *mexD* transcript in Pa1, Pa14, Pa21, Pa22 and Pa24 could not be detected either due to extremely low amounts or absence of expression of the respective pumps in these two sets of isolates (Singh et al., 2017).

Semi-quantitative RT-PCR results for *mexF* and porin *oprD* genes as evidenced by the observation of bands on agarose gel were hardly discernible and therefore, difficult to interpret even in the reference strain. Hence, their relative

transcriptional levels were evaluated using real time RT-PCR (Table 4.5). The levels of expression of *mexF* in all of the isolates were comparatively far lower than that obtained for the reference strain. In the case of *oprD*, a previous study reported reduced transcriptional levels of the gene to be considered significant when it was ≤70% compared to that of their reference strain (Xavier et al., 2010). In the present study, *oprD* remained downregulated in as many as 89.7% (n=26) of the isolates. Gene expression of *oprD* in Pa1, Pa11 and Pa19 were nearly comparable to that found in PAO strain.

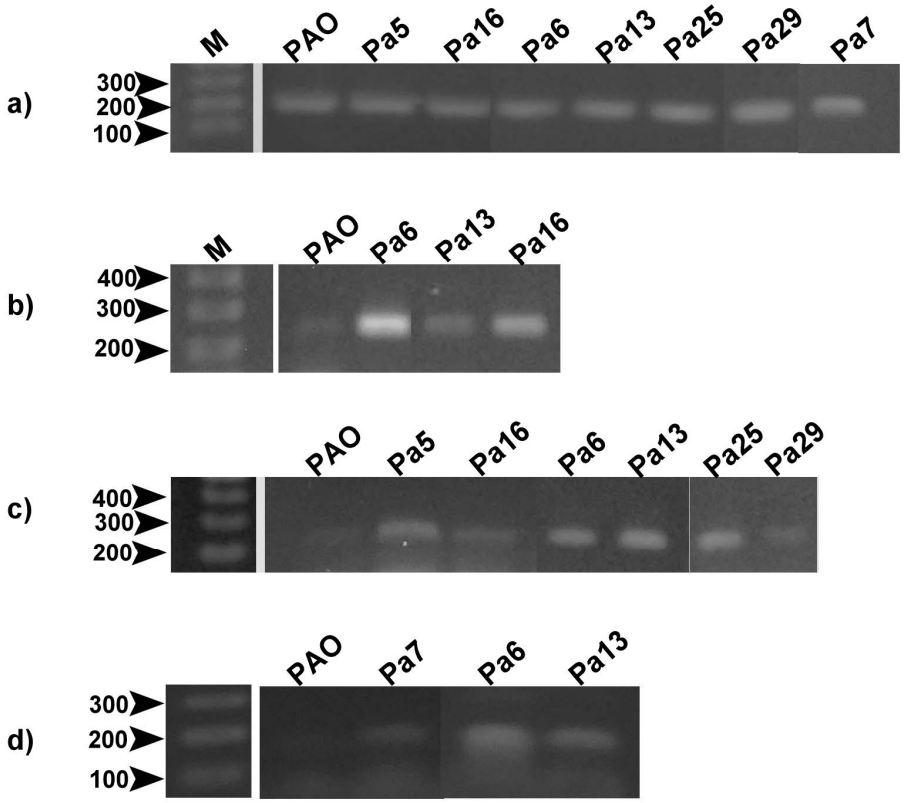


Fig. 4.12. Semi-quantitative RT-PCR. *Lane denoted M represents 100bp DNA ladder. Other lanes show P. aeruginosa MTCC reference strain PAO and clinical isolates. a) Expression of housekeeping gene, rpsL in PAO and six isolates, Pa5, Pa16, Pa6, Pa13, Pa25, Pa29 and Pa7. b) Expression of mexB in PAO and three isolates, Pa6, Pa13 and Pa16. c) Expression of mexY in PAO and six isolates, Pa5, Pa16, Pa6, Pa13, Pa25 and Pa29. d) Expression of mexD in PAO and three isolates, Pa7, Pa6 and Pa13.*

Table 4.5. Relative quantification of mRNA from *mexB, mexY*, *mexD, mexF* and *oprD*

Isolates	Relative expression*				
	mexB	***mexY***	***mexD***	***mexF***	***oprD***
Pa1	1.31	2.08	ND	0.01	0.77
Pa2	0.14	1.36	0.98	0.02	**0.12**
Pa3	0.21	0.75	0.13	0.0	**0.08**
Pa4	0.35	1.65	0.12	0.13	**0.07**
Pa5	1.19	**24.42**	0.15	0.23	**0.10**
Pa6	**11.08**	**9.65**	**14.42**	0.45	**ND**
Pa7	0.42	0.61	**4.08**	0.01	**0.05**
Pa8	0.12	2.04	0.94	0.01	**ND**
Pa9	0.13	2.01	0.35	0.01	**0.02**
Pa10	0.22	1.97	0.85	0.13	**0.29**
Pa11	1.14	2.51	1.30	0.23	1.43
Pa12	0.12	1.34	0.27	0.04	**0.16**
Pa13	**5.02**	**26.17**	**8.17**	0.0	**ND**
Pa14	0.72	0.89	ND	0.0	**ND**
Pa15	0.65	0.66	0.03	0.0	**0.05**
Pa16	**5.46**	**3.61**	0.02	0.0	**ND**
Pa17	0.29	0.27	0.29	0.01	**0.03**
Pa18	ND	0.15	0.66	0.0	**ND**
Pa19	2.11	2.17	1.42	0.15	1.05
Pa20	1.49	1.72	0.59	0.04	**0.21**
Pa21	0.11	1.58	ND	0.23	**0.09**
Pa22	0.56	1.99	ND	0.03	**ND**
Pa23	0.82	0.80	2.12	0.03	**0.13**
Pa24	1.36	1.30	ND	0.01	**ND**
Pa25	0.28	**11.79**	0.06	0.57	**ND**
Pa26	0.25	0.13	2.11	0.23	**0.48**
Pa27	0.56	1.49	0.08	0.03	**0.08**
Pa28	ND	0.21	0.0	0.04	**0.09**
Pa29	0.34	**4.08**	0.81	0.03	**0.06**

ND, not detected. **Relative to expression level in the reference strain PAO, assigned with a value of 1.0. Expression of efflux pump and porin encoding genes >3 and ≤0.7 fold respectively as compared to reference are indicated in boldface*

The 26 isolates with downregulated porin expression were found to possess a MAR index of 1.0 or less. Of these, seven isolates were found to show efflux pump activity, but only three, Pa7 (MexD overproducer), Pa16 (co-producer of both MexB and MexY) and Pa25 (MexY overproducer), were found to display a MAR index of 1.0. Notably, among the porin downregulators, three isolates, Pa20, Pa24 and Pa26,

failed to express both beta-lactamase and efflux pump activity but still had a MAR index of 1.0. Interestingly, the isolates Pa1, Pa11 and Pa19 where porin expression levels were found comparable to that of the reference strain, displayed a MAR index less than 1.0. Taken together, this clearly indicates that in these isolates, as expected, other drug resistance mechanisms are operational.

Incidentally, the present study also revealed that all isolates hyperexpressing Mex efflux pump systems were found to be devoid of ESBL production. AmpC and MBL production were observed in Mex efflux pump overproducers - Pa7, Pa13, Pa16, Pa25, and Pa29, whilst Pa6 (coproducer of MexB, MexY and MexD) and Pa5 (MexY overproducer) were devoid of beta-lactamase enzymes such as AmpC and MBL. Notably, two isolates Pa1 and Pa2, wherein efflux pump activity was phenotypically confirmed by EPI-based microplate assay, tested negative for the presence of efflux pump-related transcripts under study. This perhaps may be due to the presence of efflux pumps other than the ones studied here or may result from antibiotic-induced oxidative stress (Dridi et al., 2015; Van Acker et al., 2016). MexR repressor which negatively regulates MexAB-OprM efflux pump has been reported to function as an oxidative stress sensor. Under conditions of oxidative stress, including that induced by the antibiotic itself, MexR protein is known to dissociate from the promoter thereby inducing the operon leading to expression of efflux pump proteins (Chen et al., 2008).

4.4.4 Mutational variations and phylogenetic analyses of regulatory genes - *mexR*, *nalC* and *nalD* of *mexAB-oprM* operon

Regulatory gene sequences *mexR*, *nalC* and *nalD* of isolates with overexpressed *mexB* gene were analysed for mutations. Table 4.6 includes a complete list of mutations encountered in the present study. Genomic-PCR was carried out to amplify *mexR*, *nalC* and *nalD* genes individually producing amplicons of 729 bp, 813 bp and 788 bp respectively (Fig. 4.13).

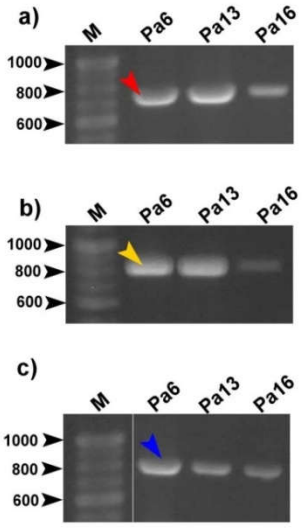


Fig. 4.13. Agarose gel (0.8%) showing mexR, nalC and nalD gene-specific amplicons from genomic-PCR of P. aeruginosa clinical isolates. *Lane denoted M represents 100bp DNA ladder; other lanes show amplicons from P. aeruginosa clinical isolates Pa6, Pa13 and Pa16. a) Red arrowhead indicates the 729 bp mexR amplicon whilst orange in b) marks the 813bp nalC amplicon and blue in c) denotes the 788 bp nalD amplicon.*

MexAB overproducers designated Pa13 and Pa16 were found to harbour mutations in their repressor genes - *mexR*, *nalC* and *nalD*. A combination of both transitional and transversional point mutations, either silent or leading to changes in amino acid substitutions were observed in *mexR* and *nalC* of Pa13 and Pa16 and in *nalD* of Pa16. However, *nalD* of Pa13 displayed only silent mutations, whilst Pa6 was conspicuous by the absence of mutations of either type. Pa13 isolate was found to possess a transversional point mutation, T→A at 377th position (^{126}valine→ glutamic acid substitution) in *mexR* gene which has already been reported previously and was considered as insignificant (Ziha-Zarifi et al., 1999; Quale et al., 2006; Choudhury et al., 2016). Two non-synonymous mutations, C→T at 20th position (^{7}proline→leucine substitution) and C→A at 427th position (^{143}proline→threonine substitution), were observed in *mexR* of Pa16 isolate, which are novel and have not yet been reported in NCBI database. This study also recorded yet another interesting novel alteration, in *mexR* of Pa16 at the 443-444 position, of an *ochre* codon to that for serine, followed by a triplet insertion for cysteine and a stop codon of *opal* type. Mutation at the 7th amino acid position of MexR in Pa16 was close to the N- terminal while other mutations were confined to the extreme end of the C-

terminal region of the repressor protein (Lim et al., 2002; Suman et al., 2006; Wilke et al., 2008). Mutations at 71st (glycine→glutamic acid) and 209th (serine→arginine) amino acid positions of *nalC* gene were reported as 'nonsense' with no effect on MexAB-OprM pump expression by Pan et al. (2016); a similar mutation was detected in *nalC* of Pa13. A transition of G→A at 212th nucleotide position (^{71}glycine→glutamic acid) was also found in *nalC* of Pa16. The *nalD* of Pa16 was found to harbour a mutation at 153rd amino acid position (leucine→glutamine). Mutation was detected within the ligand binding domain of NalD (Chen et al., 2016). Pa6 was noticeable by the absence of mutations of either type. It would be pertinent to note here that MexAB overexpression is reported to be regulated by various factors other than *mexR, nalC* and *nalD* (Maseda et al., 2004; Tian et al., 2016). The *mexR, nalC* and *nalD* gene-encoding DNA sequences of Pa6, Pa13 and Pa16 are given in Table 4.7 with the non-synonymous mutations highlighted in green colour.

Table 4.6. Summary of genetic analyses of mutations in *mexR*, *nalC* and *nalD* gene of *P. aeruginosa* clinical isolates

Isolates	***mexR* mutation**	
	Nucleotide	**Aminoacid**
Pa13	^{15}G→C, ^{18}T→C, ^{33}C→T, ^{96}A→G, ^{264}C→T, ^{327}G→A, ^{384}G→A ^{377}T→A	- ^{126}Valine→Glutamic acid
Pa16	^{20}C→T ^{201}G→A, ^{441}T→A ^{427}C→A ^{443,444}AA→CT Insertion after 444bp (TGCTGA)	^{7}Proline→Leucine - ^{143}Proline→Threonine ^{148}Stop codon→Serine Cysteine, Stop codon
	***nalC* mutation**	
	Nucleotide	**Aminoacid**
Pa13	^{212}G→A ^{258}G→A, ^{417}G→A, ^{441}C→T, ^{447}T→C ^{625}A→C	^{71}Glycine→ Glutamic acid - ^{209}Serine→Arginine
Pa16	^{212}G→A ^{595}A→T	^{71}Glycine→Glutamic acid -
	***nalD* mutation**	
	Nucleotide	**Aminoacid**
Pa13	^{78}G→A, ^{168}C→T, ^{297}G→A, ^{555}T→C	-
Pa16	^{458}T→A ^{555}T→C	^{153}Leucine→Glutamine -

'-' represents no amino acid change.

Table 4.7. DNA sequences* of *mexR*, *nalC* and *nalD* genes from three isolates Pa6, Pa13 and Pa16

Isolates	*mexR* sequences
Pa6	**Partial sequence** ATGAACTACCCCGTGAATCCCGACCTGATGCCCGCGCTGATGGCGGTCTTCCAGCATGTG 60 CGGACGCGCATCCAGAGCGAGCTCGATTGCCAGCGACTCGACCTGACCCCGCCCGACGTC 120 CATGTATTGAAGCTTATCGACGAACAACGCGGGCTGAACCTGCAGGACCTGGGACGCCAG 180 ATGTGCCGCGACAAGGCACTGATCACCCGGAAGATCCGCGAGCTGGAGGGAAGAAACCTG 240 GTCCGCCGCGAGCGCAACCCCAGCGACCAGCGCAGCTTCCAGCTCTTCCTCACCGACGAG 300 GGGCTGGCCATCCACCAGCATGCGGAGGCCATCATGTCACGCGTGCATGACGAGTTGTTT 360 GCCCCGCTCACCCCGGTGGAACAGGC 386
Pa13	**Partial sequence** ATGAACTACCCCGTCAACCCCGACCTGATGCCTGCGCTGATGGCGGTCTTCCAGCATGTG 60 CGGACGCGCATCCAGAGCGAGCTCGATTGCCAGCGGCTCGACCTGACCCCGCCCGACGTC 120 CATGTATTGAAGCTTATCGACGAACAACGCGGGCTGAACCTGCAGGACCTGGGACGCCAG 180 ATGTGCCGCGACAAGGCACTGATCACCCGGAAGATCCGCGAGCTGGAGGGAAGAAACCTG 240 GTCCGCCGCGAGCGCAACCCCAGTGACCAGCGCAGCTTCCAGCTCTTCCTCACCGACGAG 300 GGGCTGGCCATCCACCAGCATGCGGAAGCCATCATGTCACGCGTGCATGACGAGTTGTTT 360 GCCCCGCTCACCCCGGAGGAACAAGCCACCCTGGTGCATCTCCTCGACC 409
Pa16	**Complete sequence** ATGAACTACCCCGTGAATCTCGACCTGATGCCCGCGCTGATGGCGGTCTTCCAGCATGTG 60 CGGACGCGCATCCAGAGCGAGCTCGATTGCCAGCGACTCGACCTGACCCCGCCCGACGTC 120 CATGTATTGAAGCTTATCGACGAACAACGCGGGCTGAACCTGCAGGACCTGGGACGCCAG 180 ATGTGCCGCGACAAGGCACTAATCACCCGGAAGATCCGCGAGCTGGAGGGAAGAAACCTG 240 GTCCGCCGCGAGCGCAACCCCAGCGACCAGCGCAGCTTCCAGCTCTTCCTCACCGACGAG 300 GGGCTGGCCATCCACCAGCATGCGGAGGCCATCATGTCACGCGTGCATGACGAGTTGTTT 360 GCCCCGCTCACCCCGGTGGAACAGGCCACCCTGGTGCATCTCCTCGACCAGTGCCTGGCC 420 GCGCAAACGCTTGAGGATATATCTTGCTGA 450
Isolates	***nalC* sequences**
Pa6	**Complete sequence** ATGAACGATGCTTCTCCCCGTCTGACCGAACGCGGCAGGCAACGCCGCCGCGCCATGCTC 60 GACGCCGCTACCCAGGCCTTTCTCGAACACGGTTTCGAAGGCACCACCCTGGACATGGTG 120 ATAGAACGGGCCGGTGGTTCACGGGGGACCCTGTACAGCTCCTTCGGCGGCAAGGAGGGC 180 CTGTTCGCCGCGGTGATCGCCCACATGATCGGGGAAATCTTCGACGACAGCGCCGATCAG 240 CCGCGCCCCGCCGCCACGCTGAGCGCCACCCTCGAGCATTTCGGCCGGCGCTTTCTCACC 300 AGCCTGCTCGATCCCCGCTGCCAGAGCCTCTATCGCCTGGTGGTGGCGGAATCCCCGCGG 360 TTTCCGGCGATCGGCAAGTCCTTCTACGAGCAGGGGCCGCAGCAGAGCTATCTGCTGCTC 420 AGCGAGCGACTGGCCGCGGTCGCTCCTCACATGGACGAGGAAACGCTCTACGCGGTGGCC 480 TGCCAGTTTCTCGAGATGCTCAAGGCCGACCTGTTCCTCAAGGCCCTCAGCGTGGCCGAC 540 TTCCAGCCGACCATGGCGCTGCTGGAAACCCGCCTCAAGCTGTCGGTGGACATCATCGCC 600 TGCTACCTGGAACACCTGTCGCAGAGCCCCGCGCAGGGCTGA 642
Pa13	**Complete sequence** ATGAACGATGCTTCTCCCCGTCTGACCGAACGCGGCAGGCAACGCCGCCGCGCCATGCTC 60 GACGCCGCTACCCAGGCCTTTCTCGAACACGGTTTCGAAGGCACCACCCTGGACATGGTG 120 ATAGAACGGGCCGGTGGTTCACGGGGGACCCTGTACAGCTCCTTCGGCGGCAAGGAGGGC 180 CTGTTCGCCGCGGTGATCGCCCACATGATCGAGGAAATCTTCGACGACAGCGCCGATCAG 240 CCGCGCCCCGCCGCCACACTGAGCGCCACCCTCGAGCATTTCGGCCGGCGCTTTCTCACC 300 AGCCTGCTCGATCCCCGCTGCCAGAGCCTCTATCGCCTGGTGGTGGCGGAATCCCCGCGG 360 TTTCCGGCGATCGGCAAGTCCTTCTACGAGCAGGGGCCGCAGCAGAGCTATCTGCTACTC 420 AGCGAGCGACTGGCCGCGGTTGCTCCCCACATGGACGAGGAAACGCTCTACGCGGTGGCC 480 TGCCAGTTTCTCGAGATGCTCAAGGCCGACCTGTTCCTCAAGGCCCTCAGCGTGGCCGAC 540 TTCCAGCCGACCATGGCGCTGCTGGAAACCCGCCTCAAGCTGTCGGTGGACATCATCGCC 600 TGCTACCTGGAACACCTGTCGCAGCGCCCCGCGCAGGGCTGA 642

Pa16	**Partial sequence** ATGAACGATGCTTCTCCCCGTCTGACCGAACGCGGCAGGCAACGCCGCCGCGCCATGCTC **60** GACGCCGCTACCCAGGCCTTTCTCGAACACGGTTTCGAAGGCACCACCCTGGACATGGTG **120** ATAGAACGGGCCGGTGGTTCACGGGGGACCCTGTACAGCTCCTTCGGCGGCAAGGAGGGC **180** CTGTTCGCCGCGGTGATCGCCCACATGATCGAGGAAATCTTCGACGACAGCGCCGATCAG **240** CCGCGCCCCGCCGCCACGCTGAGCGCCACCCTCGAGCATTTCGGCCGGCGCTTTCTCACC **300** AGCCTGCTCGATCCCCGCTGCCAGAGCCTCTATCGCCTGGTGGTGGCGGAATCCCCGCGG **360** TTTCCGGCGATCGGCAAGTCCTTCTACGAGCAGGGGCCGCAGCAGAGCTATCTGCTGCTC **420** AGCGAGCGACTGGCCGCGGTCGCTCCTCACATGGACGAGGAAACGCTCTACGCGGTGGCC **480** TGCCAGTTTCTCGAGATGCTCAAGGCCGACCTGTTCCTCAAGGCCCTCAGCGTGGCCGAC **540** TTCCAGCCGACCATGGCGCTGCTGGAAACCCGCCTCAAGCTGTCGGTGGACATCT **595**
Isolates	***nalD* sequences**
Pa6	**Complete sequence** ATGCGACGCACAAAGGAAGATTCTGAAAAAACCCGTACGGCCATCCTCCTGGCCGCCGAG **60** GAACTGTTCCTGGAAAAGGGCGTGTCCCATACCAGCCTGGAACAGATCGCCAGGGCCGCC **120** GGGGTGACCCGTGGCGCCGTCTACTGGCACTTCCAGAACAAGGCCCACCTGTTCAACGAG **180** ATGCTCAACCAGGTACGCCTGCCGCCGGAGCAACTCACCGAGCGCCTGTCCGGCTGCGAT **240** GGCAGCGACCCGCTGCGCTCGCTCTACGACCTCTGCCTGGAGGCCGTGCAATCGTTGCTG **300** ACGCAGGAGAAGAAGCGCCGCATCCTGACCATCCTGATGCAACGTTGCGAATTCACCGAG **360** GAACTGCGCGAGGCGCAGGAACGCAACAACGCCTTCGTGCAGATGTTCATCGAACTCTGC **420** GAGCAGTTGTTCGCCCGCGACGAATGCCGTGTGCGGCTGCATCCGGGCATGACCCCGAGG **480** ATCGCCTCGCGCGCCTTGCACGCGCTGATCCTGGGCCTGTTCAACGACTGGTTGCGCGAC **540** CCGCGCCTGTTCGATCCGGATACGGACGCGGAACACCTGCTGGAGCCGATGTTCCGTGGC **600** CTGGTGCGCGACTGGGGTCAGGCCAGCTCGGCGCCGTAG **639**
Pa13	**Complete sequence** ATGCGACGCACAAAGGAAGATTCTGAAAAAACCCGTACGGCCATCCTCCTGGCCGCCGAG **60** GAACTGTTCCTGGAAAAAGGCGTGTCCCATACCAGCCTGGAACAGATCGCCAGGGCCGCC **120** GGGGTGACCCGTGGCGCCGTCTACTGGCACTTCCAGAACAAGGCCCATCTGTTCAACGAG **180** ATGCTCAACCAGGTACGCCTGCCGCCGGAGCAACTCACCGAGCGCCTGTCCGGCTGCGAT **240** GGCAGCGACCCGCTGCGCTCGCTCTACGACCTCTGCCTGGAGGCCGTGCAATCGTTACTG **300** ACGCAGGAGAAGAAGCGCCGCATCCTGACCATCCTGATGCAACGTTGCGAATTCACCGAG **360** GAACTGCGCGAGGCGCAGGAACGCAACAACGCCTTCGTGCAGATGTTCATCGAACTCTGC **420** GAGCAGTTGTTCGCCCGCGACGAATGCCGTGTGCGGCTGCATCCGGGCATGACCCCGAGG **480** ATCGCCTCGCGCGCCTTGCACGCGCTGATCCTGGGCCTGTTCAACGACTGGTTGCGCGAC **540** CCGCGCCTGTTCGACCCGGATACGGACGCGGAACACCTGCTGGAGCCGATGTTCCGTGGC **600** CTGGTGCGCGACTGGGGTCAGGCCAGCTCGGCGCCGTAG **639**
Pa16	**Complete sequence** ATGCGACGCACAAAGGAAGATTCTGAAAAAACCCGTACGGCCATCCTCCTGGCCGCCGAG **60** GAACTGTTCCTGGAAAAGGGCGTGTCCCATACCAGCCTGGAACAGATCGCCAGGGCCGCC **120** GGGGTGACCCGTGGCGCCGTCTACTGGCACTTCCAGAACAAGGCCCACCTGTTCAACGAG **180** ATGCTCAACCAGGTACGCCTGCCGCCGGAGCAACTCACCGAGCGCCTGTCCGGCTGCGAT **240** GGCAGCGACCCGCTGCGCTCGCTCTACGACCTCTGCCTGGAGGCCGTGCAATCGTTGCTG **300** ACGCAGGAGAAGAAGCGCCGCATCCTGACCATCCTGATGCAACGTTGCGAATTCACCGAG **360** GAACTGCGCGAGGCGCAGGAACGCAACAACGCCTTCGTGCAGATGTTCATCGAACTCTGC **420** GAGCAGTTGTTCGCCCGCGACGAATGCCGTGTGCGGCAGCATCCGGGCATGACCCCGAGG **480** ATCGCCTCGCGCGCCTTGCACGCGCTGATCCTGGGCCTGTTCAACGACTGGTTGCGCGAC **540** CCGCGCCTGTTCGACCCGGATACGGACGCGGAACACCTGCTGGAGCCGATGTTCCGTGGC **600** CTGGTGCGCGACTGGGGTCAGGCCAGCTCGGCGCCGTAG **639**

*****Non-synonymous mutations are highlighted with green colour and insertion mutation in silver colour.*

Sequences obtained from *mexR*, *nalC*, and *nalD* of three MexB overproducing isolates, Pa6, Pa13 and Pa16, were concatenated as a prelude to the phylogenetic study. The resultant composite sequence was then subjected to Bayesian phylogenetic analysis along with a select group of nine closely related sequences deposited in the NCBI database. The nine sequences were chosen on the basis of differences in geographical areas of their report, highest query coverage and minimum 'E' values, when subjected to BLASTN analysis with the sequences obtained from PCR amplicons in this study. *P. aeruginosa* strain, PAO1 (GenBank accession no. AE004091.2), was taken as the outgroup for the purpose (Jochumsen et al., 2016). The best fit model of sequence evolution based on AIC was detected as HKY for *mexR* and *nalC* and as HKY+I for *nalD*. Potential scale reduction factor (PSRF) value assessed by MrBayes was observed to be 1.0 and the estimated sample size (ESS) was above 100. Phylogenetic analysis of three loci - *mexR*, *nalC* and *nalD* - concatenated, composite sequence (Fig. 4.14) revealed that the sequences from isolate Pa6 formed a distinct lineage. Interestingly, the sequence from Pa13 showed genetic similarity with that of MDR *P. aeruginosa* -VRFPA04 (GenBank accession no. CP008739.2), obtained from a keratitis patient from a tertiary eye care center in Tamil Nadu, India (Murugan et al., 2016) and the homology was also evident with the sequences deposited from Taiwan (GenBank accession no. CP004061.1). Interestingly, the concatenated sequence of Pa16 isolate exhibited similarity with the sequence of *P. aeruginosa* first reported with *bla*NDM-1 in North America (GenBank accession no. CP012901.1) which was colistin non-susceptible, isolated from an elderly Canadian patient who was directly transferred to Calgary hospital, Alberta, Canada, following a prolonged hospital stay in New Delhi, India (Mataseje et al., 2016). The two sequences reported from Brazil (GenBank accession no. CP021380.1) and Malaysia (GenBank accession no. CP007147.1) were found to be clustered into a distinct clade.

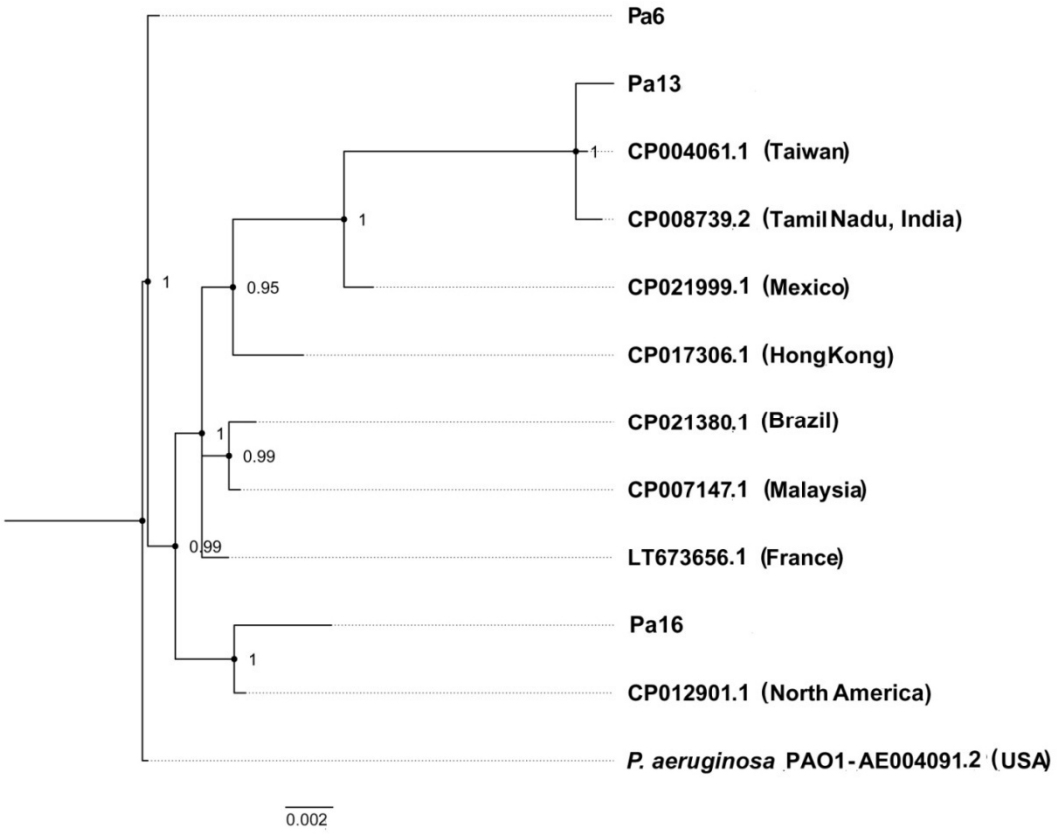


Fig. 4.14. Bayesian phylogenetic tree reconstructed from three loci of P.aeruginosa – mexR, nalC and nalD*. Concatenated alignment drawn to scale with the branch lengths representing evolutionary distance. Scale bar denotes probability of nucleotide change. Analysis was run for 1,000,000 generations. Clade credibility values are shown at each node. P.aeruginosa, PAO1, was used as the outgroup.*

4.4.5 Mutational variations and phylogenetic analysis of regulatory gene- *mexZ* of *mexXY* operon

Genomic-PCR was carried out to amplify the *mexZ* gene producing an amplicon of 1000 bp in isolates with overexpressed *mexY* gene (Fig. 4.15). Sequences obtained from these amplicons showed a variety of silent/point mutations (Table 4.8 and 4.9). However, two isolates, Pa16 and Pa29, were found to display point mutations in *mexZ* gene, leading to substitution of alanine by threonine at the 2nd and cysteine for tyrosine at the 52nd amino acid position respectively. These isolates showed mutations in the DNA binding domain at the N-terminal of MexZ

(Alguel et al., 2010; Jahandideh, 2013). It may be noted that point mutations in *mexZ* gene of the two isolates, which perhaps might be responsible for upregulation of MexXY observed in this study were found to be identical positionally to those reported in GenBank accession nos. CP012901.1 (Mataseje et al. 2016) and WP_023123846.1 respectively. PCR amplification of *mexZ* in Pa6, which showed a 9.65 fold higher level of MexY compared to that of PAO strain, was unsuccessful despite several attempts with different primer sets and cycling conditions. A similar case of amplification failure has also been reported by Poonsuk et al. (2014).

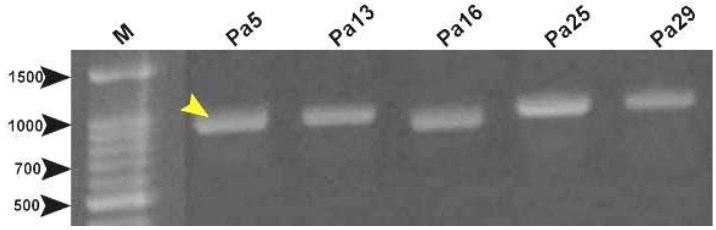


Fig. 4.15. Agarose gel (0.8%) showing mexZ gene-specific amplicons from genomic-PCR of P. aeruginosa clinical isolates. *Lane denoted M represents 100bp DNA ladder; other lanes show amplicons from P. aeruginosa clinical isolates Pa5, Pa13, Pa16, Pa25 and Pa29. Yellow arrowhead indicates the 1000bp mexZ amplicon.*

Table 4.8. Summary of genetic analyses of mutations in *mexZ* gene of *P. aeruginosa* clinical isolates

Isolates	*mexZ* mutation	
	Nucleotide	**Aminoacid**
Pa5	^{93}C→T, ^{210}C→T, ^{357}C→T, ^{367}T→C, ^{438}A→G	-
Pa13	^{93}C→T, ^{231}C→T	-
Pa16	^{4}G→A	^{2}Alanine→Threonine
Pa25	^{93}C→T, ^{438}A→G, ^{576}C→T, ^{585}C→A	-
Pa29	^{155}A→G ^{382}C→T, ^{438}A→G	^{52}Tyrosine→Cysteine -

'-' represents no amino acid change.

Table 4.9. DNA sequences*of *mexZ* gene from five isolates Pa5, Pa13, Pa16, Pa25 and Pa29

Isolates	*mexZ* sequences	
Pa5	**Complete sequence**	
	GTGGCCAGGAAAACCAAAGAGGAATCCCAGAAAACCCGCGACGGCATACTCGATGCCGCC	60
	GAGCGGGTTTTCCTGGAAAAGGGCGTGGGCACTACTGCCATGGCCGACCTGGCGGACGCC	120
	GCCGGGGTTTCTCGCGGTGCGGTCTACGGCCACTACAAGAACAAGATCGAGGTCTGCCTG	180
	GCGATGTGCGACCGCGCCTTCGGCCAGATTGAGGTACCCGACGAAAACGCCAGGGTGCCG	240
	GCGCTGGATATCCTCCTGCGCGCCGGCATGGGCTTTCTCCGCCAGTGCTGCGAGCCCGGT	300
	TCGGTGCAGCGGGTGCTGGAGATCCTCTACCTCAAGTGCGAACGCAGCGACGAGAATGAG	360
	CCGCTGCTGCGCCGCCGCGAGCTGCTCGAGAAGCAGGGGCAACGCTTCGGCCTCCGGCAG	420
	ATCCGCCGGGCGGTGGAGCGCGGCGAACTGCCGGCGCGGCTGGACGTCGAGCTGGCCAGC	480
	ATCTATCTGCAATCGCTCTGGGACGGCATCTGCGGCACCCTGGCCTGGACCGAGCGCCTG	540
	CGCGACGATCCCTGGAACCGCGCCGAACGCATGTTCCGCGCCGGCCTCGACAGCCTGCGC	600
	AGTTCTCCCTACCTGTTGCTGGCGGACGCCTGA	633
Pa13	**Partial sequence**	
	GTGGCCAGGAAAACCAAAGAGGAATCCCAGAAAACCCGCGACGGCATACTCGATGCCGCC	60
	GAGCGGGTTTTCCTGGAAAAGGGCGTGGGCACTACTGCCATGGCCGACCTGGCGGACGCC	120
	GCCGGGGTTTCTCGCGGTGCGGTCTACGGCCACTACAAGAACAAGATCGAGGTCTGCCTG	180
	GCGATGTGCGACCGCGCCTTCGGCCAGATCGAGGTACCCGACGAAAACGCTAGGGTGCCG	240
	GCGCTGGATATCCTCCTGCGCGCCGGCATGGGCTTTCTCCGCCAGTGCTGCGAGCCCGGT	300
	TCGGTGCA	308
Pa16	**Partial sequence**	
	GTGACCAGGAAAACCAAAGAGGAATCCCAGAAAACCCGCGACGGCATACTCGATGCCGCC	60
	GAGCGGGTTTTCCTGGAAAAGGGCGTGGGCACCACTGCCATGGCCGACCTGGCGGACGCC	120
	GCCGGGGTTTCTCGCGGTGCGGTCTACGGCCACTACAAGAACAAGATCGAGGTCTGCCTG	180
	GCGATGTGCGACCGCGCCTTCGGCCAGATCGAGGTACCCGACGAAAACGCCAGGGTGCCG	240
	GCGCTGGATATCCTCCTGCGCGCCGGCATGGGCTTTCTCCGCCAGTGCTGCGAGCCCGGT	300
	TCGGTGCAGCGGGTGCTGGAGATCCTCTACCTCAAGTGCGAACGCAGCGACGAGAACGAG	360
	CCGCTGTTGCGCCGCCGCGAGCTGCTCGAGAAGCAGGGGCAACGCTTCGGCCTCCGGCAG	420
	ATCCGCCGG	429
Pa25	**Complete sequence**	
	GTGGCCAGGAAAACCAAAGAGGAATCCCAGAAAACCCGCGACGGCATACTCGATGCCGCC	60
	GAGCGGGTTTTCCTGGAAAAGGGCGTGGGCACTACTGCCATGGCCGACCTGGCGGACGCC	120
	GCCGGGGTTTCTCGCGGTGCGGTCTACGGCCACTACAAGAACAAGATCGAGGTCTGCCTG	180
	GCGATGTGCGACCGCGCCTTCGGCCAGATCGAGGTACCCGACGAAAACGCCAGGGTGCCG	240
	GCGCTGGATATCCTCCTGCGCGCCGGCATGGGCTTTCTCCGCCAGTGCTGCGAGCCCGGT	300
	TCGGTGCAGCGGGTGCTGGAGATCCTCTACCTCAAGTGCGAACGCAGCGACGAGAACGAG	360
	CCGCTGTTGCGCCGCCGCGAGCTGCTCGAGAAGCAGGGGCAACGCTTCGGCCTCCGGCAG	420
	ATCCGCCGGGCGGTGGAGCGCGGCGAACTGCCGGCGCGGCTGGACGTCGAGCTGGCCAGC	480
	ATCTATCTGCAATCGCTCTGGGACGGCATCTGCGGCACCCTGGCCTGGACCGAGCGCCTG	540
	CGCGACGATCCCTGGAACCGCGCCGAACGCATGTTTCGCGCCGGACTCGACAGCCTGCGC	600
	AGTTCTCCCTACCTGTTGCTGGCGGACGCCTGA	633
Pa29	**Partial sequence**	

	----------CACTGCCATGGCCGACCTGGCGGACGCC	28
	GCCGGGGTTTCTCGCGGTGCGGTCTACGGCCACTGCAAGAACAAGATCGAGGTCTGCCTG	88
	GCGATGTGCGACCGCGCCTTCGGCCAGATCGAGGTACCCGACGAAAACGCCAGGGTGCCG	148
	GCGCTGGATATCCTCCTGCGCGCCGGCATGGGCTTTCTCCGCCAGTGCTGCGAGCCCGGT	208
	TCGGTGCAGCGGGTGCTGGAGATCCTCTACCTCAAGTGCGAACGCAGCGACGAGAACGAG	268
	CCGCTGTTGCGCCGCCGCGAGTTGCTCGAGAAGCAGGGGCAACGCTTCGGCCTCCGGCAG	328
	ATCCGCCGGGCGGTGGAGCGCGGCGAACTGCCGGCGCGGCTGGACGTCGAGCTGGCCAGC	388
	ATCTATCTGCAATCGCTCTGGGACGGCATCTGCGGCACCCTGGCCTGGACCGAGCGCCTG	448
	CGCGACGATCCCTGGAACCGCGCCGAACGCATGTTCCGCGCCGGCCTCGACAGCCTGCGC	508
	AGTTCTCCCTACCTGTTGCTGGCGGACGCCTGA	541

**Non-synonymous mutations are highlighted with green colour*

The *mexZ* sequences from five MexY overproducer strains - Pa5, Pa13, Pa16, Pa25 and Pa29 were clubbed together with a set of nine other similar sequences retrieved from the GenBank database for construction of a phylogram using *P. aeruginosa* strain, PAO1 (GenBank accession no. AE004091.2) as the outgroup. The best fit model of sequence evolution based on AIC was detected as GTR+I. PSRF value assessed by MrBayes was observed to be 1.0 and the ESS was above 100. But the phylogram generated employing mexZ sequence resulted in low clade credibility values (< 0.3) and hence was considered to be non-significant (Figure not shown).

4.4.6 Mutational variations and phylogenetic analysis of regulatory gene *nfxB* of *mexCD-oprJ* operon

Genomic-PCR was carried out to amplify *nfxB* gene producing amplicons of 750 bp in three MexCD-OprJ overproducers - Pa6, Pa7 and Pa13 (Fig. 4.16). DNA sequences obtained from these showed various synonymous and non-synonymous mutations (Table 4.10 and 4.11). Mutation analysis revealed point mutation, T → C at 239th position (^{80}leucine → serine substitution) in Pa6, T→C at 349th position (^{117}serine→proline substitution) in Pa7 and C→T at 14th position (^{5}serine→ phenylalanine substitution) in Pa13. Notably, mutations detected in Pa6 and Pa7 isolates have been found to be novel and have not yet been reported in GenBank data base, whilst the mutation present in Pa13 was found to be similar to that already reported by Murugan et al. (2016).

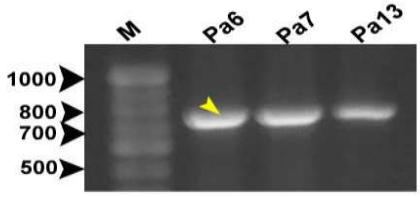


Fig. 4.16. Agarose gel (0.8%) showing nfxB gene-specific amplicons from genomic-PCR of P. aeruginosa clinical isolates. *Lane denoted M represents 100bp DNA ladder and other lanes show amplicons from isolates Pa6, Pa7and Pa13. Yellow arrowhead indicates the 750 bp nfxB amplicon.*

Table 4.10. Summary of genetic analyses of mutations in *nfxB* gene of *P. aeruginosa* clinical isolates

Isolates	*nfxB* mutation	
	Nucleotide	Aminoacid
Pa6	^{239}T→C ^{555}T→G	^{80}Leucine → Serine -
Pa7	^{39}G→A,^{183}A→G,^{423}G→A,^{480}T→C,^{486}A→T,^{537}T→C, ^{543}C→T,^{555}T→G ^{349}T→C	- ^{117}Serine→Proline
Pa13	^{14}C→T ^{135}C→T,^{141}G→A,^{555}T→G	^{5}Serine→Phenylalnine -

'-' represents no amino acid change.

Table 4.11. DNA sequences*of *nfxB* gene from three isolates Pa6, Pa7 and Pa13

Isolates	*nfxB* sequences	
Pa6	**Complete sequence**	
	ATGACCCTGATTTCCCATGACGAGCGACTCATCAAGGCGCTGGCAGTCGCTATCGTCGAC	60
	CGCCCGCGAGCGACGCTGAAGGAACTGGCCGAGGCGGCCGGCGTAAGCAAGGCCACCCTG	120
	CACCGCTTCTGCGGCACGCGGGACAACCTGGTGCAGATGCTCGAGGACCACGGAGAGACC	180
	GTACTGAACCAGATCATCCAGGCCTGCGACCTGGAGCATGCCGAGCCTCTGGAGGCGTCG	240
	CAGCGCCTGATCAAGGAACACCTCACCCACCGCGAGCTGCTGGTATTCCTGGTATTCCAG	300
	TACCGCCCGGACTTCCTCGACCCGCACGGCGAAGGCGCACGCTGGCAGTCCTACCTGGAA	360
	GCGCTGGACGCCTTCTTCCTGCGCGGACAGCAGAAAGGCGTGTTTCGCATCGACATCACG	420
	GCGGCCGTGTTCACCGAACTGTTCATCACCCTGGTCTACGGCATGGTCGATGCGGAACGT	480
	CGCGGACGGGCGGCCAGCTCCAATTCCGCGCATACCCTGGAGCAGATGTTCCTCCATGGC	540
	GCCTCCAATCCGGCGCGCTCCTGA	564
Pa7	**Complete sequence**	
	ATGACCCTGATTTCCCATGACGAGCGACTCATCAAGGCACTGGCAGTCGCTATCGTCGAC	60
	CGCCCGCGAGCGACGCTGAAGGAACTGGCCGAGGCGGCCGGCGTAAGCAAGGCCACCCTG	120
	CACCGCTTCTGCGGCACGCGGGACAACCTGGTGCAGATGCTCGAGGACCACGGAGAGACC	180
	GTGCTGAACCAGATCATCCAGGCCTGCGACCTGGAGCATGCCGAGCCTCTGGAGGCGTTG	240
	CAGCGCCTGATCAAGGAACACCTCACCCACCGCGAGCTGCTGGTATTCCTGGTATTCCAG	300
	TACCGCCCGGACTTCCTCGACCCGCACGGCGAAGGCGCACGCTGGCAGCCCTACCTGGAA	360
	GCGCTGGACGCCTTCTTCCTGCGCGGACAGCAGAAAGGCGTGTTTCGCATCGACATCACG	420
	GCAGCCGTGTTCACCGAACTGTTCATCACCCTGGTCTACGGCATGGTCGATGCGGAACGC	480
	CGCGGTCGGGCGGCCAGCTCCAATTCCGCGCATACCCTGGAGCAGATGTTCCTCCACGGC	540
	GCTTCCAATCCGGCGCGCTCCTGA	564
Pa13	**Complete sequence**	
	ATGACCCTGATTTTCCATGACGAGCGACTCATCAAGGCGCTGGCAGTCGCTATCGTCGAC	60
	CGCCCGCGAGCGACGCTGAAGGAACTGGCCGAGGCGGCCGGCGTAAGCAAGGCCACCCTG	120
	CACCGCTTCTGCGGTACGCGAGACAACCTGGTGCAGATGCTCGAGGACCACGGAGAGACC	180
	GTACTGAACCAGATCATCCAGGCCTGCGACCTGGAGCATGCCGAGCCTCTGGAGGCGTTG	240
	CAGCGCCTGATCAAGGAACACCTCACCCACCGCGAGCTGCTGGTATTCCTGGTATTCCAG	300
	TACCGCCCGGACTTCCTCGACCCGCACGGCGAAGGCGCACGCTGGCAGTCCTACCTGGAA	360
	GCGCTGGACGCCTTCTTCCTGCGCGGACAGCAGAAAGGCGTGTTTCGCATCGACATCACG	420
	GCGGCCGTGTTCACCGAACTGTTCATCACCCTGGTCTACGGCATGGTCGATGCGGAACGT	480
	CGCGGACGGGCGGCCAGCTCCAATTCCGCGCATACCCTGGAGCAGATGTTCCTCCATGGC	540
	GCCTCCAATCCGGCGCGCTCCTGA	564

**Non-synonymous mutations are highlighted with green colour*

Phylogram (Fig. 4.17) was constructed using three *nfxB* gene sequences of MexCD-OprJ overproducers - Pa6, Pa7 and Pa13 and nine other selected sequences of *P. aeruginosa* isolates retrieved from the GenBank database including the reference strain, *P. aeruginosa* - PAO1(GenBank accession no. AE004091.2). For this analysis, *Pseudomonas chlororaphis* (GenBank accession no. CP011110.1) was taken as the outgroup as this sequence showed better sequence similarity with a higher query coverage - unlike that carried out with respect to regulatory genes of m*exAB-oprM* operon using PAO1 sequence as the outgroup. GTR was selected as the best fit model of sequence evolution on the basis of AIC. PSRF and ESS value were observed as 1.0 and above 100 respectively. Analysis was run for 1,000,000 generations and clade credibility values represented at each node were found to be > 0.5. Bayesian phylogenetic analysis revealed that the sequence from Pa6 was found to exhibit similarity with isolates from Taiwan (GenBank accession no. CP004061.1), Mexico (GenBank accession no. CP021999.1) and Brazil (GenBank accession no. CP021380.1). Pa13 isolate showed similarity with that reported from Tamil Nadu (CP008739.2) whilst Pa7 was found to be similar to *P. aeruginosa* sequences deposited from North America (CP012901.1).

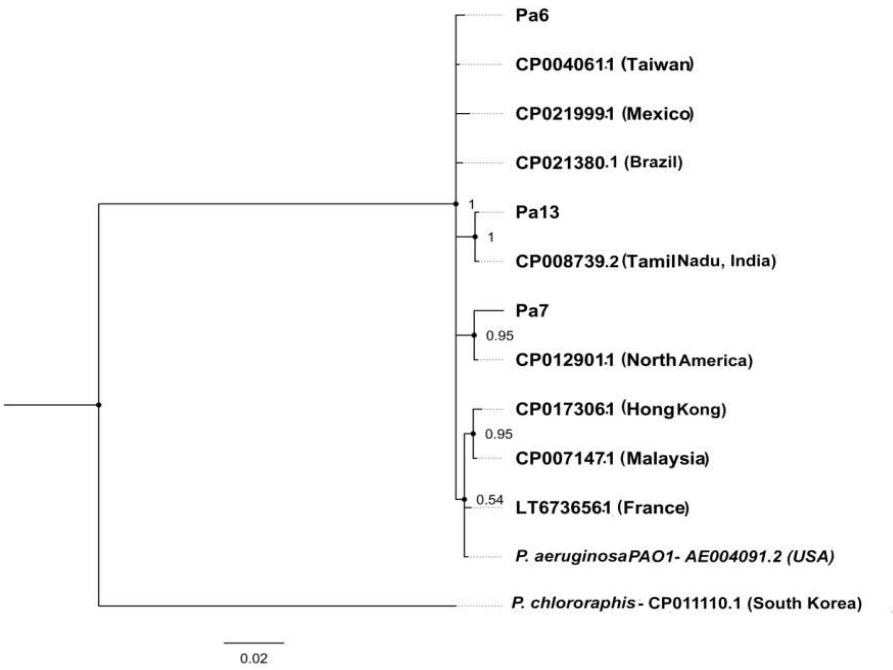


Fig. 4.17. Bayesian phylogenetic tree reconstructed employing nfxB ampliconic sequences from P.aeruginosa isolates. *Scale bar denotes probability of nucleotide change. Analysis was run for 1,000,000 generations. Clade credibility values are shown at each node. P. chlororaphis (GenBank accession no. CP011110.1) was used as the outgroup.*

4.4.7 Mutational variations and phylogenetic analysis of regulatory gene *mexT* of *mexEF-oprN* operon and *oprD*

The gene *mexT* encodes a LysR-type transcriptional activator protein, MexT, which mediates hyperexpression of MexEF-OprN efflux pump in *nfxC*-type mutants resistant to quinolones (Maseda et al., 2000). Expression of MexEF-OprN is usually linked with OprD porin down regulation mediated by the regulatory action of the MexT (Köhler et al., 1997). However, in this study involving 29 selected isolates (section 4.4.3), 26 strains with downregulated OprD failed to amplify the *mexF* transcript beyond the levels of the MTCC reference strain as evidenced by the results obtained with real time RT-PCR analyses. Hence, *mexT* was amplified in all of the 26 isolates as it also serves as the regulatory gene of the *oprD* operon. Amplicons were obtained in 24 out of 26 isolates except in Pa2 and Pa6. Primers used for amplification were confined to the N-terminal portion encoded by the 5' end of the *mexT* gene, which included the 8 bp insert involved in inactivating the transcriptional activator. The amplicon size was found to be 650 bp (Fig. 4.18).

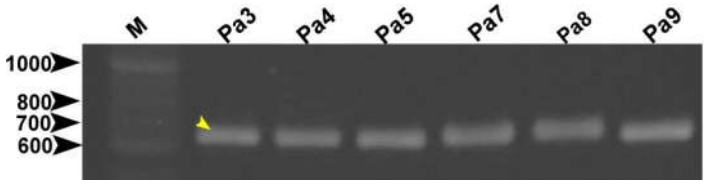


Fig. 4.18. Agarose gel (0.8%) showing mexT gene-specific amplicons from genomic-PCR of P. aeruginosa clinical isolates. *Lane denoted M represents 100bp DNA ladder. Other lanes show amplicons from representatives of P. aeruginosa isolates Pa3, Pa4, Pa5, Pa7, Pa8 and Pa9. Yellow arrowhead indicates the 650 bp amplicon.*

Interestingly, a comparative analysis of DNA sequences of the amplicons with that of *P. aeruginosa*-PAO1 revealed occurrence of an 8-bp deletion (GGCCAGCC) at nucleotide position 235 within a 14-bp direct repeat in all isolates including reference *P. aeruginosa* MTCC- PAO strain (Table 4.12 and 4.13). A similar type of deletion at identical genomic locations in different *P.aeruginosa* strains have been reported previously by many researchers (Morita et al., 2015; Wright et al., 2015; Mataseje et al., 2016; Murugan et al., 2016). Likewise,

Ocampo–Sosa *et al.* (2012) discovered an 8-bp deletion (GCCGGCCA) at position 240; still another 8-bp deletion (CGGCCAGC) at 226th nucleotide position has also been reported in other independent studies (Maseda et al., 2000; Singh et al., 2017).

Table 4.12. Summary of mutations observed in *mexT* amplicons from *P. aeruginosa* clinical isolates

Isolates	*mexT* mutation	
	Nucleotide	**Aminoacid**
Pa3	^{235}GGCCAGCC deletion (^{321}G→A, ^{444}C→A,^{514}T→A)*	Frameshift
Pa4	^{235}GGCCAGCC deletion ^{514}T→A*	Frameshift
Pa5	^{235}GGCCAGCC deletion (^{472}T→C,^{501}G→A, ^{514}T→A)*	Frameshift
Pa7	^{178}C→T ^{235}GGCCAGCC deletion (^{372}T→C, ^{472}T→C, ^{501}G→A, ^{514}T→A)*	^{60}Proline→Serine Frameshift
Pa8	^{235}GGCCAGCC deletion (^{321}G→A, ^{444}C→A, ^{514}T→A)*	Frameshift
Pa9	^{235}GGCCAGCC deletion (^{472}T→C, ^{501}G→A, ^{514}T→A)*	Frameshift
Pa10	^{178}C→T ^{235}GGCCAGCC deletion (^{321}G→A, ^{468}C→T, ^{514}T→A)*	^{60}Proline→Serine Frame shift
Pa12	^{178}C→T ^{235}GGCCAGCC deletion (^{372}T→C, ^{393}T→C, ^{514}T→A)*	^{60}Proline→Serine Frameshift
Pa13	^{235}GGCCAGCC deletion (^{252}C→T, ^{427}G→A, ^{514}T→A)*	Frameshift
Pa14	^{178}C→T ^{235}GGCCAGCC deletion (^{472}T→C, ^{501}G→A, ^{514}T→A)*	^{60}Proline→Serine Frame shift
Pa15	^{178}C→T ^{235}GGCCAGCC deletion (^{321}G→A, ^{472}T→C, ^{501}G→A, ^{514}T→A)*	^{60}Proline→Serine Frameshift
Pa16	^{178}C→T ^{235}GGCCAGCC deletion (^{278}T→C, ^{321}G→A, ^{468}C→T, ^{514}T→A)*	^{60}Proline→Serine Frameshift
Pa17	^{235}GGCCAGCC deletion (^{472}T→C, ^{501}G→A, ^{514}T→A)*	Frameshift
Pa18	^{197}G→A ^{235}GGCCAGCC deletion ^{514}T→A*	^{66}Arginine→Histidine Frame shift
Pa20	^{235}GGCCAGCC deletion (^{252}C→T, ^{372}T→C, ^{393}T→C, ^{465}C→T, ^{514}T→A)*	Frameshift

Pa21	^{235}GGCCAGCC deletion (^{252}C→T, ^{427}G→A, ^{514}T→A)*	Frameshift
Pa22	^{235}GGCCAGCC deletion (^{252}C→T, ^{372}T→C, ^{393}T→C, ^{465}C→T, ^{514}T→A)*	Frameshift
Pa23	^{178}C→T ^{235}GGCCAGCC deletion (^{321}G→A, ^{468}C→T, ^{514}T→A)*	^{60}Proline→Serine Frameshift
Pa24	^{235}GGCCAGCC deletion (^{472}T→C, ^{501}G→A, ^{514}T→A)*	Frameshift
Pa25	^{178}C→T ^{235}GGCCAGCC deletion (^{321}G→A, ^{514}T→A)*	^{60}Proline→Serine Frameshift
Pa26	^{178}C→T ^{235}GGCCAGCC deletion (^{372}T→C, ^{472}T→C, ^{501}G→A, ^{514}T→A)*	^{60}Proline→Serine Frameshift
Pa27	^{178}C→T ^{235}GGCCAGCC deletion (^{321}G→A, ^{514}T→A)*	^{60}Proline→Serine Frameshift
Pa28	^{235}GGCCAGCC deletion (^{472}T→C, ^{501}G→A, ^{514}T→A)*	Frameshift
Pa29	^{178}C→T ^{235}GGCCAGCC deletion (^{321}G→A, ^{472}T→C, ^{501}G→A, ^{514}T→A)*	^{60}Proline→Serine Frameshift

**Due to change in the reading frame consequent to 8 bp deletion, changes in amino acids occurring past the frameshift mutation are not listed*

Table 4.13. DNA sequences*of *mexT* gene from twenty four isolates of *P. aeruginosa*

Isolates	mexT mutation
Pa3	**Partial sequence** ----------ATGCCCCTCCGGCACCTCGCCAGGCCCCGCCCCGTCTCGCAC 42 GCAAGGCTTGACGGCGAGCCCCCGCGGTTGCAGCCTCTAGCCCCTGGAAACGAGGAACGC 102 CATGAACCGAAACGACCTGCGCCGCGTCGATCTGAACCTGCTGATCGTGTTCGAGACCCT 162 GATGCACGAACGCAGCGTGACCCGCGCCGCAGAGAAACTGTTCCTCGGCCAGCC------ 216 --GGCCATCAGCGCCGCGCTGTCGCGCCTGCGCACGCTGTTCGACGACCCGCTGTTCGTC 274 CGTACCGGACGCAGCATGGAACCCACCGCGCGAGCCCAGGAAATCTTCGCCCACCTGTCG 334 CCGGCGCTGGATTCCATCTCCACCGCCATGAGTCGCGCCAGCGAGTTCGATCCGGCGACC 394 AGCACCGCGGTGTTCCGCATCGGACTTTCCGACGACGTCGAGTTCGGCCTGTTGCCGCCC 454 CTGCTCCGCCGCCTGCGCGCGGAGGCGCCGGGGATCGTCCTCGTCGTGCGCCGCGCCAAC 514 TATCTATTGATGCCGAACCTGCTGGCCTCGGGGGAGATCTCGGTGGGCGTCAGCTACAC- 573
Pa4	**Partial sequence** ----------ATCCTATGCCCCTCCGGCACCTCGCCAGGCCCCGCCCCGTCTCGCAC 47 GCAAGGCTTGACGGCGAGCCCCCGCGGTTGCAGCCTCTAGCCCCTGGAAACGAGGAACGC 107 CATGAACCGAAACGACCTGCGCCGCGTCGATCTGAACCTGCTGATCGTGTTCGAGACCCT 167 GATGCACGAACGCAGCGTGACCCGCGCCGCAGAGAAACTGTTCCTCGGCCAGCC------ 221 --GGCCATCAGCGCCGCGCTGTCGCGCCTGCGCACGCTGTTCGACGACCCGCTGTTCGTC 279 CGTACCGGACGCAGCATGGAGCCCACCGCGCGAGCCCAGGAAATCTTCGCCCACCTGTCG 339 CCGGCGCTGGATTCCATCTCCACCGCCATGAGTCGCGCCAGCGAGTTCGATCCGGCGACC 399 AGCACCGCGGTGTTCCGCATCGGCCTTTCCGACGACGTCGAGTTCGGCCTGTTGCCGCCC 459 CTGCTCCGCCGCCTGCGCGCGGAGGCGCCGGGGATCGTCCTCGTCGTGCGCCGCGCCAAC 519 TATCTATTGATGCCGAACCTGCTGGCCTCGGGGGAGATCTCGGTGGGCGTCAGCTACACC 579 GACGAACT---------- 587

Pa5	**Partial sequence** ------------------ATGCCCCTCCGGCACCTCGCCAGGCCCCGCCCCGTCTCGCAC 42 GCAAGGCTTGACGGCGAGCCCCCGCGGTTGCAGCCTCTAGCCCCTGGAAACGAGGAACGC 102 CATGAACCGAAACGACCTGCGCCGCGTCGATCTGAACCTGCTGATCGTGTTCGAGACCCT 162 GATGCACGAACGCAGCGTGACCCGCGCCGCAGAGAAACTGTTCCTCGGCCAGCC------ 216 --GGCCATCAGCGCCGCGCTGTCGCGCCTGCGCACGCTGTTCGACGACCCGCTGTTCGTC 274 CGTACCGGACGCAGCATGGAGCCCACCGCGCGAGCCCAGGAAATCTTCGCCCACCTGTCG 334 CCGGCGCTGGATTCCATCTCCACCGCCATGAGTCGCGCCAGCGAGTTCGATCCGGCGACC 394 AGCACCGCGGTGTTCCGCATCGGCCTTTCCGACGACGTCGAGTTCGGCCTGCTGCCGCCC 454 CTGCTCCGCCGCCTGCGCGCAGAGGCGCCGGGGATCGTCCTCGTCGTGCGCCGCGCCAAC 514 TATCTATTGATGCCGAACCTGCTGGCCTCGGGGGAGATCTCGGTGGGCGTCAGCTAC--- 571
Pa7	**Partial sequence** ------------------ATGCCCCTCCGGCACCTCGCCAGGCCCCGCCCCGTCTCGCAC 42 GCAAGGCTTGACGGCGAGCCCCCGCGGTTGCAGCCTCTAGCCCCTGGAAACGAGGAACGC 102 CATGAACCGAAACGACCTGCGCCGCGTCGATCTGAACCTGCTGATCGTGTTCGAGACTCT 162 GATGCACGAACGCAGCGTGACCCGCGCCGCAGAGAAACTGTTCCTCGGCCAGCC------ 216 --GGCCATCAGCGCCGCGCTGTCGCGCCTGCGCACGCTGTTCGACGACCCGCTGTTCGTC 274 CGTACCGGACGCAGCATGGAGCCCACCGCGCGAGCCCAGGAAATCTTCGCCCACCTGTCG 334 CCGGCGCTGGACTCCATCTCCACCGCCATGAGTCGCGCCAGCGAGTTCGATCCGGCGACC 394 AGCACCGCGGTGTTCCGCATCGGCCTTTCCGACGACGTCGAGTTCGGCCTGCTGCCGCCC 454 CTGCTCCGCCGCCTGCGCGCAGAGGCGCCGGGGATCGTCCTCGTCGTGCGCCGCGCCAAC 514 TATCTATTGATGCCGAACCTGCTGGCCTCGGGGGAGATCTCGGTGGGCGTCAGCTACACC 574 GACGAACTG---------- 583
Pa8	**Partial sequence** ------------------ATGCCCCTCCGGCACCTCGCCAGGCCCCGCCCCGTCTCGCAC 42 GCAAGGCTTGACGGCGAGCCCCCGCGGTTGCAGCCTCTAGCCCCTGGAAACGAGGAACGC 102 CATGAACCGAAACGACCTGCGCCGCGTCGATCTGAACCTGCTGATCGTGTTCGAGACCCT 162 GATGCACGAACGCAGCGTGACCCGCGCCGCAGAGAAACTGTTCCTCGGCCAGCC------ 216 --GGCCATCAGCGCCGCGCTGTCGCGCCTGCGCACGCTGTTCGACGACCCGCTGTTCGTC 274 CGTACCGGACGCAGCATGGAACCCACCGCGCGAGCCCAGGAAATCTTCGCCCACCTGTCG 334 CCGGCGCTGGATTCCATCTCCACCGCCATGAGTCGCGCCAGCGAGTTCGATCCGGCGACC 394 AGCACCGCGGTGTTCCGCATCGGACTTTCCGACGACGTCGAGTTCGGCCTGTTGCCGCCC 454 CTGCTCCGCCGCCTGCGCGCGGAGGCGCCGGGGATCGTCCTCGTCGTGCGCCGCGCCAAC 514 TATCTATTGATGCCGAACCTGCTGGCCTCGGGGGAGATCTCGGTGGGCGTCAG------- 567
Pa9	**Partial sequence** ------------------ATGCCCCTCCGGCACCTCGCCAGGCCCCGCCCCGTCTCGCAC 42 GCAAGGCTTGACGGCGAGCCCCCGCGGTTGCAGCCTCTAGCCCCTGGAAACGAGGAACGC 102 CATGAACCGAAACGACCTGCGCCGCGTCGATCTGAACCTGCTGATCGTGTTCGAGACCCT 162 GATGCACGAACGCAGCGTGACCCGCGCCGCAGAGAAACTGTTCCTCGGCCAGCC------ 216 --GGCCATCAGCGCCGCGCTGTCGCGCCTGCGCACGCTGTTCGACGACCCGCTGTTCGTC 274 CGTACCGGACGCAGCATGGAGCCCACCGCGCGAGCCCAGGAAATCTTCGCCCACCTGTCG 334 CCGGCGCTGGATTCCATCTCCACCGCCATGAGTCGCGCCAGCGAGTTCGATCCGGCGACC 394 AGCACCGCGGTGTTCCGCATCGGCCTTTCCGACGACGTCGAGTTCGGCCTGCTGCCGCCC 454 CTGCTCCGCCGCCTGCGCGCAGAGGCGCCGGGGATCGTCCTCGTCGTGCGCCGCGCCAAC 514 TATCTATTGATGCCGAACCTGCTGGCCTCGGGGGAGATCTCGGTGGGCGTCAGCTAC--- 571
Pa10	**Partial sequence** ------------------ATGCCCCTCCGGCACCTCGCCAGGCCCCGCCCCGTCTCGCAC 42 GCAAGGCTTGACGGCGAGCCCCCGCGGTTGCAGCCTCTAGCCCCTGGAAACGAGGAACGC 102 CATGAACCGAAACGACCTGCGCCGCGTCGATCTGAACCTGCTGATCGTGTTCGAGACTCT 162 GATGCACGAACGCAGCGTGACCCGCGCCGCAGAGAAACTGTTCCTCGGCCAGCC------ 216 --GGCCATCAGCGCCGCGCTGTCGCGCCTGCGCACGCTGTTCGACGACCCGCTGTTCGTC 274 CGTACCGGACGCAGCATGGAACCCACCGCGCGAGCCCAGGAAATCTTCGCCCACCTGTCG 334 CCGGCGCTGGATTCCATCTCCACCGCCATGAGTCGCGCCAGCGAGTTCGATCCGGCGACC 394 AGCACCGCGGTGTTCCGCATCGGCCTTTCCGACGACGTCGAGTTCGGTCTGTTGCCGCCC 454 CTGCTCCGCCGCCTGCGCGCGGAGGCGCCGGGGATCGTCCTCGTCGTGCGCCGCGCCAAC 514 TATCTATTGATGCCGAACCTGCTGGCCTCGGGGGAGATCTCGGTGGGCGTCAGCTACAC- 573

Pa12	**Partial sequence** ------------------ATGCCCCTCCGGCACCTCGCCAGGCCCCGCCCCGTCTCGCAC 42 GCAAGGCTTGACGGCGAGCCCCCGCGGTTGCAGCCTCTAGCCCCTGGAAACGAGGAACGC 102 CATGAACCGAAACGACCTGCGCCGCGTCGATCTGAACCTGCTGATCGTGTTCGAGACTCT 162 GATGCACGAACGCAGCGTGACCCGCGCCGCAGAGAAACTGTTCCTCGGCCAGCC------ 216 --GGCCATCAGCGCCGCGCTGTCGCGCCTGCGCACGCTGTTCGACGACCCGCTGTTCGTC 274 CGTACCGGACGCAGCATGGAGCCCACCGCGCGAGCCCAGGAAATCTTCGCCCACCTGTCG 334 CCGGCGCTGGACTCCATCTCCACCGCCATGAGCCGCGCCAGCGAGTTCGATCCGGCGACC 394 AGCACCGCGGTGTTCCGCATCGGCCTTTCCGACGACGTCGAGTTCGGCCTGTTGCCGCCC 454 CTGCTCCGCCGCCTGCGCGCGGAGGCGCCGGGGATCGTCCTCGTCGTGCGCCGCGCCAAC 514 TATCTATTGATGCCGAACCTGCTGGCCTCGGGGGAGATCTCGGTGGGCGTCAGCTACAC- 573
Pa13	**Partial sequence** ------------------ATGCCCCTCCGGCACCTCGCCAGGCCCCGCCCCGTCTCGCAC 42 GCAAGGCTTGACGGCGAGCCCCCGCGGTTGCAGCCTCTAGCCCCTGGAAACGAGGAACGC 102 CATGAACCGAAACGACCTGCGCCGCGTCGATCTGAACCTGCTGATCGTGTTCGAGACCCT 162 GATGCACGAACGCAGCGTGACCCGCGCCGCAGAGAAACTGTTCCTCGGCCAGCC------ 216 --GGCCATCAGTGCCGCGCTGTCGCGCCTGCGCACGCTGTTCGACGACCCGCTGTTCGTC 274 CGTACCGGACGCAGCATGGAGCCCACCGCGCGAGCCCAGGAAATCTTCGCCCACCTGTCG 334 CCGGCGCTGGATTCCATCTCCACCGCCATGAGTCGCGCCAGCGAGTTCGATCCGGCGACC 394 AGCACCACGGTGTTCCGCATCGGCCTTTCCGACGACGTCGAGTTCGGCCTGTTGCCGCCC 454 CTGCTCCGCCGCCTGCGCGCGGAGGCGCCGGGGATCGTCCTCGTCGTGCGCCGCGCCAAC 514 TATCTATTGATGCCGAACCTGCTGGCCTCGGGGGAGATCTCGGTGGGCGTCAGCTACAC- 573
Pa14	**Partial sequence** ------------------ATGCCCCTCCGGCACCTCGCCAGGCCCCGCCCCGTCTCGCAC 42 GCAAGGCTTGACGGCGAGCCCCCGCGGTTGCAGCCTCTAGCCCCTGGAAACGAGGAACGC 102 CATGAACCGAAACGACCTGCGCCGCGTCGATCTGAACCTGCTGATCGTGTTCGAGACTCT 162 GATGCACGAACGCAGCGTGACCCGCGCCGCAGAGAAACTGTTCCTCGGCCAGCC------ 216 --GGCCATCAGCGCCGCGCTGTCGCGCCTGCGCACGCTGTTCGACGACCCGCTGTTCGTC 274 CGTACCGGACGCAGCATGGAGCCCACCGCGCGAGCCCAGGAAATCTTCGCCCACCTGTCG 334 CCGGCGCTGGATTCCATCTCCACCGCCATGAGTCGCGCCAGCGAGTTCGATCCGGCGACC 394 AGCACCGCGGTGTTCCGCATCGGCCTTTCCGACGACGTCGAGTTCGGCCTGCTGCCGCCC 454 CTGCTCCGCCGCCTGCGCGCAGAGGCGCCGGGGATCGTCCTCGTCGTGCGCCGCGCCAAC 514 TATCTATTGATGCCGAACCTGCTGGCCTCGGGGGAGATCTCGGTGGGCGTCAGCTAC--- 571
Pa15	**Partial sequence** ------------------ATGCCCCTCCGGCACCTCGCCAGGCCCCGCCCCGTCTCGCAC 42 GCAAGGCTTGACGGCGAGCCCCCGCGGTTGCAGCCTCTAGCCCCTGGAAACGAGGAACGC 102 CATGAACCGAAACGACCTGCGCCGCGTCGATCTGAACCTGCTGATCGTGTTCGAGACTCT 162 GATGCACGAACGCAGCGTGACCCGCGCCGCAGAGAAACTGTTCCTCGGCCAGCC------ 216 --GGCCATCAGCGCCGCGCTGTCGCGCCTGCGCACGCTGTTCGACGACCCGCTGTTCGTC 274 CGTACCGGACGCAGCATGGAACCCACCGCGCGAGCCCAGGAAATCTTCGCCCACCTGTCG 334 CCGGCGCTGGATTCCATCTCCACCGCCATGAGTCGCGCCAGCGAGTTCGATCCGGCGACC 394 AGCACCGCGGTGTTCCGCATCGGCCTTTCCGACGACGTCGAGTTCGGCCTGCTGCCGCCC 454 CTGCTCCGCCGCCTGCGCGCAGAGGCGCCGGGGATCGTCCTCGTCGTGCGCCGCGCCAAC 514 TATCTATTGATGCCGAACCTGCTGGCCTCGGGGGAGATCTCGGTGGGCGTCAGCTACAC- 573
Pa16	**Partial sequence** ------------------ATGCCCCTCCGGCACCTCGCCAGGCCCCGCCCCGTCTCGCAC 42 GCAAGGCTTGACGGCGAGCCCCCGCGGTTGCAGCCTCTAGCCCCTGGAAACGAGGAACGC 102 CATGAACCGAAACGACCTGCGCCGCGTCGATCTGAACCTGCTGATCGTGTTCGAGACTCT 162 GATGCACGAACGCAGCGTGACCCGCGCCGCAGAGAAACTGTTCCTCGGCCAGCC------ 216 --GGCCATCAGCGCCGCGCTGTCGCGCCTGCGCACGCCGTTCGACGACCCGCTGTTCGTC 274 CGTACCGGACGCAGCATGGAACCCACCGCGCGAGCCCAGGAAATCTTCGCCCACCTGTCG 334 CCGGCGCTGGATTCCATCTCCACCGCCATGAGTCGCGCCAGCGAGTTCGATCCGGCGACC 394 AGCACCGCGGTGTTCCGCATCGGCCTTTCCGACGACGTCGAGTTCGGTCTGTTGCCGCCC 454 CTGCTCCGCCGCCTGCGCGCGGAGGCGCCGGGGATCGTCCTCGTCGTGCGCCGCGCCAAC 514 TATCTATTGATGCCGAACCTGCTGGCCTCGGGGGAGATCTCGGTGGGCGTCAGCTACACC 574 GACGAACTG---------- 583

Pa17	**Partial sequence**	
	----------ATGCCCCTCCGGCACCTCGCCAGGCCCCGCCCCGTCTCGCAC	**42**
	GCAAGGCTTGACGGCGAGCCCCCGCGGTTGCAGCCTCTAGCCCCTGGAAACGAGGAACGC	**102**
	CATGAACCGAAACGACCTGCGCCGCGTCGATCTGAACCTGCTGATCGTGTTCGAGACCCT	**162**
	GATGCACGAACGCAGCGTGACCCGCGCCGCAGAGAAACTGTTCCTCGGCCAGCC------	**216**
	--GGCCATCAGCGCCGCGCTGTCGCGCCTGCGCACGCTGTTCGACGACCCGCTGTTCGTC	**274**
	CGTACCGGACGCAGCATGGAGCCCACCGCGCGAGCCCAGGAAATCTTCGCCCACCTGTCG	**334**
	CCGGCGCTGGATTCCATCTCCACCGCCATGAGTCGCGCCAGCGAGTTCGATCCGGCGACC	**394**
	AGCACCGCGGTGTTCCGCATCGGCCTTTCCGACGACGTCGAGTTCGGCCTGCTGCCGCCC	**454**
	CTGCTCCGCCGCCTGCGCGCAGAGGCGCCGGGGATCGTCCTCGTCGTGCGCCGCGCCAAC	**514**
	TATCTATTGATGCCGAACCTGCTGGCCTCGGGGGAGATCTCGGTGGGCGTCAGCTACACC	**574**
	GA----------	**576**
Pa18	**Partial sequence**	
	----------ATGCCCCTCCGGCACCTCGCCAGGCCCCGCCCCGTCTCGCAC	**42**
	GCAAGGCTTGACGGCGAGCCCCCGCGGTTGCAGCCTCTAGCCCCTGGAAACGAGGAACGC	**102**
	CATGAACCGAAACGACCTGCGCCGCGTCGATCTGAACCTGCTGATCGTGTTCGAGACCCT	**162**
	GATGCACGAACGCAGCATGACCCGCGCCGCAGAGAAACTGTTCCTCGGCCAGCC------	**216**
	--GGCCATCAGCGCCGCGCTGTCGCGCCTGCGCACGCTGTTCGACGACCCGCTGTTCGTC	**274**
	CGTACCGGACGCAGCATGGAGCCCACCGCGCGAGCCCAGGAAATCTTCGCCCACCTGTCG	**334**
	CCGGCGCTGGATTCCATCTCCACCGCCATGAGTCGCGCCAGCGAGTTCGATCCGGCGACC	**394**
	AGCACCGCGGTGTTCCGCATCGGCCTTTCCGACGACGTCGAGTTCGGCCTGTTGCCGCCC	**454**
	CTGCTCCGCCGCCTGCGCGCGGAGGCGCCGGGGATCGTCCTCGTCGTGCGCCGCGCCAAC	**514**
	TATCTATTGATGCCGAACCTGCTGGCCTCGGGGGAGATCTCGGTGGGCGTCAGCTACAC-	**573**
Pa20	**Partial sequence**	
	----------ATGCCCCTCCGGCACCTCGCCAGGCCCCGCCCCGTCTCGCAC	**42**
	GCAAGGCTTGACGGCGAGCCCCCGCGGTTGCAGCCTCTAGCCCCTGGAAACGAGGAACGC	**102**
	CATGAACCGAAACGACCTGCGCCGCGTCGATCTGAACCTGCTGATCGTGTTCGAGACCCT	**162**
	GATGCACGAACGCAGCGTGACCCGCGCCGCAGAGAAACTGTTCCTCGGCCAGCC------	**216**
	--GGCCATCAGTGCCGCGCTGTCGCGCCTGCGCACGCTGTTCGACGACCCGCTGTTCGTC	**274**
	CGTACCGGACGCAGCATGGAGCCCACCGCGCGAGCCCAGGAAATCTTCGCCCACCTGTCG	**334**
	CCGGCGCTGGACTCCATCTCCACCGCCATGAGCCGCGCCAGCGAGTTCGATCCGGCGACC	**394**
	AGCACCGCGGTGTTCCGCATCGGCCTTTCCGACGACGTCGAGTTTGGCCTGTTGCCGCCC	**454**
	CTGCTCCGCCGCCTGCGCGCGGAGGCGCCGGGGATCGTCCTCGTCGTGCGCCGCGCCAAC	**514**
	TATCTATTGATGCCGAACCTGCTGGCCTCGGGGGAGATCTCGGTGGGCGTCAGCTACAC-	**573**
Pa21	**Partial sequence**	
	----------ATGCCCCTCCGGCACCTCGCCAGGCCCCGCCCCGTCTCGCAC	**42**
	GCAAGGCTTGACGGCGAGCCCCCGCGGTTGCAGCCTCTAGCCCCTGGAAACGAGGAACGC	**102**
	CATGAACCGAAACGACCTGCGCCGCGTCGATCTGAACCTGCTGATCGTGTTCGAGACCCT	**162**
	GATGCACGAACGCAGCGTGACCCGCGCCGCAGAGAAACTGTTCCTCGGCCAGCC------	**216**
	--GGCCATCAGTGCCGCGCTGTCGCGCCTGCGCACGCTGTTCGACGACCCGCTGTTCGTC	**274**
	CGTACCGGACGCAGCATGGAGCCCACCGCGCGAGCCCAGGAAATCTTCGCCCACCTGTCG	**334**
	CCGGCGCTGGATTCCATCTCCACCGCCATGAGTCGCGCCAGCGAGTTCGATCCGGCGACC	**394**
	AGCACCACGGTGTTCCGCATCGGCCTTTCCGACGACGTCGAGTTCGGCCTGTTGCCGCCC	**454**
	CTGCTCCGCCGCCTGCGCGCGGAGGCGCCGGGGATCGTCCTCGTCGTGCGCCGCGCCAAC	**514**
	TATCTATTGATGCCGAACCTGCTGGCCTCGGGGGAGATCTCGGTGGGCGTCAGCTACAC-	**573**
Pa22	**Partial sequence**	
	----------ATGCCCCTCCGGCACCTCGCCAGGCCCCGCCCCGTCTCGCAC	**42**
	GCAAGGCTTGACGGCGAGCCCCCGCGGTTGCAGCCTCTAGCCCCTGGAAACGAGGAACGC	**102**
	CATGAACCGAAACGACCTGCGCCGCGTCGATCTGAACCTGCTGATCGTGTTCGAGACCCT	**162**
	GATGCACGAACGCAGCGTGACCCGCGCCGCAGAGAAACTGTTCCTCGGCCAGCC------	**216**
	--GGCCATCAGTGCCGCGCTGTCGCGCCTGCGCACGCTGTTCGACGACCCGCTGTTCGTC	**274**
	CGTACCGGACGCAGCATGGAGCCCACCGCGCGAGCCCAGGAAATCTTCGCCCACCTGTCG	**334**
	CCGGCGCTGGACTCCATCTCCACCGCCATGAGCCGCGCCAGCGAGTTCGATCCGGCGACC	**394**
	AGCACCGCGGTGTTCCGCATCGGCCTTTCCGACGACGTCGAGTTTGGCCTGTTGCCGCCC	**454**
	CTGCTCCGCCGCCTGCGCGCGGAGGCGCCGGGGATCGTCCTCGTCGTGCGCCGCGCCAAC	**514**
	TATCTATTGATGCCGAACCTGCTGGCCTCGGGGGAGATCTCGGTGGGCGTCAGCTAC---	**571**

Pa23	**Partial sequence** ---------AGTGATCCTATGCCCCTCCGGCACCTCGCCAGGCCCCGCCCCGTCTCGCAC 51 GCAAGGCTTGACGGCGAGCCCCCGCGGTTGCAGCCTCTAGCCCCTGGAAACGAGGAACGC 111 CATGAACCGAAACGACCTGCGCCGCGTCGATCTGAACCTGCTGATCGTGTTCGAGACTCT 171 GATGCACGAACGCAGCGTGACCCGCGCCGCAGAGAAACTGTTCCTCGGCCAGCC------ 225 --GGCCATCAGCGCCGCGCTGTCGCGCCTGCGCACGCTGTTCGACGACCCGCTGTTCGTC 283 CGTACCGGACGCAGCATGGAACCCACCGCGCGAGCCCAGGAAATCTTCGCCCACCTGTCG 343 CCGGCGCTGGATTCCATCTCCACCGCCATGAGTCGCGCCAGCGAGTTCGATCCGGCGACC 403 AGCACCGCGGTGTTCCGCATCGGCCTTTCCGACGACGTCGAGTTCGGTCTGTTGCCGCCC 463 CTGCTCCGCCGCCTGCGCGCGGAGGCGCCGGGGATCGTCCTCGTCGTGCGCCGCGCCAAC 523 TATCTATTGATGCCGAACCTGCTGGCCTCGGGGGAGATCTCGGTGGGCGTCAGCTAC--- 580
Pa24	**Partial sequence** -----------------ATGCCCCTCCGGCACCTCGCCAGGCCCCGCCCCGTCTCGCAC 42 GCAAGGCTTGACGGCGAGCCCCCGCGGTTGCAGCCTCTAGCCCCTGGAAACGAGGAACGC 102 CATGAACCGAAACGACCTGCGCCGCGTCGATCTGAACCTGCTGATCGTGTTCGAGACCCT 162 GATGCACGAACGCAGCGTGACCCGCGCCGCAGAGAAACTGTTCCTCGGCCAGCC------ 216 --GGCCATCAGCGCCGCGCTGTCGCGCCTGCGCACGCTGTTCGACGACCCGCTGTTCGTC 274 CGTACCGGACGCAGCATGGAGCCCACCGCGCGAGCCCAGGAAATCTTCGCCCACCTGTCG 334 CCGGCGCTGGATTCCATCTCCACCGCCATGAGTCGCGCCAGCGAGTTCGATCCGGCGACC 394 AGCACCGCGGTGTTCCGCATCGGCCTTTCCGACGACGTCGAGTTCGGCCTGCTGCCGCCC 454 CTGCTCCGCCGCCTGCGCGCAGAGGCGCCGGGGATCGTCCTCGTCGTGCGCCGCGCCAAC 514 TATCTATTGATGCCGAACCTGCTGGCCTCGGGGGAGATCTCGGTGGGCGTCAGCTACAC- 573
Pa25	**Partial sequence** ---------AGTGATCCTATGCCCCTCCGGCACCTCGCCAGGCCCCGCCCCGTCTCGCAC 51 GCAAGGCTTGACGGCGAGCCCCCGCGGTTGCAGCCTCTAGCCCCTGGAAACGAGGAACGC 111 CATGAACCGAAACGACCTGCGCCGCGTCGATCTGAACCTGCTGATCGTGTTCGAGACTCT 171 GATGCACGAACGCAGCGTGACCCGCGCCGCAGAGAAACTGTTCCTCGGCCAGCC------ 225 --GGCCATCAGCGCCGCGCTGTCGCGCCTGCGCACGCTGTTCGACGACCCGCTGTTCGTC 283 CGTACCGGACGCAGCATGGAACCCACCGCGCGAGCCCAGGAAATCTTCGCCCACCTGTCG 343 CCGGCGCTGGATTCCATCTCCACCGCCATGAGTCGCGCCAGCGAGTTCGATCCGGCGACC 403 AGCACCGCGGTGTTCCGCATCGGCCTTTCCGACGACGTCGAGTTCGGCCTGTTGCCGCCC 463 CTGCTCCGCCGCCTGCGCGCGGAGGCGCCGGGGATCGTCCTCGTCGTGCGCCGCGCCAAC 523 TATCTATTGATGCCGAACCTGCTGGCCTCGGGGGAGATCTCGGTGGGCGTCAGCTACACC 583 GA---------- 585
Pa26	**Partial sequence** ----------------CTATGCCCCTCCGGCACCTCGCCAGGCCCCGCCCCGTCTCGCAC 44 GCAAGGCTTGACGGCGAGCCCCCGCGGTTGCAGCCTCTAGCCCCTGGAAACGAGGAACGC 104 CATGAACCGAAACGACCTGCGCCGCGTCGATCTGAACCTGCTGATCGTGTTCGAGACTCT 164 GATGCACGAACGCAGCGTGACCCGCGCCGCAGAGAAACTGTTCCTCGGCCAGCC------ 218 --GGCCATCAGCGCCGCGCTGTCGCGCCTGCGCACGCTGTTCGACGACCCGCTGTTCGTC 276 CGTACCGGACGCAGCATGGAGCCCACCGCGCGAGCCCAGGAAATCTTCGCCCACCTGTCG 336 CCGGCGCTGGACTCCATCTCCACCGCCATGAGTCGCGCCAGCGAGTTCGATCCGGCGACC 396 AGCACCGCGGTGTTCCGCATCGGCCTTTCCGACGACGTCGAGTTCGGCCTGCTGCCGCCC 456 CTGCTCCGCCGCCTGCGCGCAGAGGCGCCGGGGATCGTCCTCGTCGTGCGCCGCGCCAAC 516 TATCTATTGATGCCGAACCTGCTGGCCTCGGGGGAGATCTCGGTGGGCGTCAGCTAC--- 573
Pa27	**Partial sequence** ---------AGTGATCCTATGCCCCTCCGGCACCTCGCCAGGCCCCGCCCCGTCTCGCAC 51 GCAAGGCTTGACGGCGAGCCCCCGCGGTTGCAGCCTCTAGCCCCTGGAAACGAGGAACGC 111 CATGAACCGAAACGACCTGCGCCGCGTCGATCTGAACCTGCTGATCGTGTTCGAGACTCT 171 GATGCACGAACGCAGCGTGACCCGCGCCGCAGAGAAACTGTTCCTCGGCCAGCC------ 225 --GGCCATCAGCGCCGCGCTGTCGCGCCTGCGCACGCTGTTCGACGACCCGCTGTTCGTC 283 CGTACCGGACGCAGCATGGAACCCACCGCGCGAGCCCAGGAAATCTTCGCCCACCTGTCG 343 CCGGCGCTGGATTCCATCTCCACCGCCATGAGTCGCGCCAGCGAGTTCGATCCGGCGACC 403 AGCACCGCGGTGTTCCGCATCGGCCTTTCCGACGACGTCGAGTTCGGCCTGTTGCCGCCC 463 CTGCTCCGCCGCCTGCGCGCGGAGGCGCCGGGGATCGTCCTCGTCGTGCGCCGCGCCAAC 523 TATCTATTGATGCCGAACCTGCTGGCCTCGGGGGAGATCTCGGTGGGCGTCAGCTAC--- 580

Pa28	**Partial sequence** ----------ATGCCCCTCCGGCACCTCGCCAGGCCCCGCCCCGTCTCGCAC 42 GCAAGGCTTGACGGCGAGCCCCCGCGGTTGCAGCCTCTAGCCCCTGGAAACGAGGAACGC 102 CATGAACCGAAACGACCTGCGCCGCGTCGATCTGAACCTGCTGATCGTGTTCGAGACCCT 162 GATGCACGAACGCAGCGTGACCCGCGCCGCAGAGAAACTGTTCCTCGGCCAGCC------ 216 --GGCCATCAGCGCCGCGCTGTCGCGCCTGCGCACGCTGTTCGACGACCCGCTGTTCGTC 274 CGTACCGGACGCAGCATGGAGCCCACCGCGCGAGCCCAGGAAATCTTCGCCCACCTGTCG 334 CCGGCGCTGGATTCCATCTCCACCGCCATGAGTCGCGCCAGCGAGTTCGATCCGGCGACC 394 AGCACCGCGGTGTTCCGCATCGGCCTTTCCGACGACGTCGAGTTCGGCCTGCTGCCGCCC 454 CTGCTCCGCCGCCTGCGCGCAGAGGCGCCGGGGATCGTCCTCGTCGTGCGCCGCGCCAAC 514 TATCTATTGATGCCGAACCTGCTGGCCTCGGGGGAGATCTCGGTGGGCGTCAGCTAC--- 571
Pa29	**Partial sequence** ----------ATGCCCCTCCGGCACCTCGCCAGGCCCCGCCCCGTCTCGCAC 42 GCAAGGCTTGACGGCGAGCCCCCGCGGTTGCAGCCTCTAGCCCCTGGAAACGAGGAACGC 102 CATGAACCGAAACGACCTGCGCCGCGTCGATCTGAACCTGCTGATCGTGTTCGAGACTCT 162 GATGCACGAACGCAGCGTGACCCGCGCCGCAGAGAAACTGTTCCTCGGCCAGCC------ 216 --GGCCATCAGCGCCGCGCTGTCGCGCCTGCGCACGCTGTTCGACGACCCGCTGTTCGTC 274 CGTACCGGACGCAGCATGGAACCCACCGCGCGAGCCCAGGAAATCTTCGCCCACCTGTCG 334 CCGGCGCTGGATTCCATCTCCACCGCCATGAGTCGCGCCAGCGAGTTCGATCCGGCGACC 394 AGCACCGCGGTGTTCCGCATCGGCCTTTCCGACGACGTCGAGTTCGGCCTGCTGCCGCCC 454 CTGCTCCGCCGCCTGCGCGCAGAGGCGCCGGGGATCGTCCTCGTCGTGCGCCGCGCCAAC 514 TATCTATTGATGCCGAACCTGCTGGCCTCGGGGGAGATCTCGGTGGGCGTCAGCTAC--- 571

**Non-synonymous mutations are highlighted in green and the 8 bp deletion in cyan colour*

Further, the results of BLASTN analyses of all *mexT* amplicon sequences against the GenBank deposits of *P. aeruginosa* have been summarized in (Table 4.14). The sole exceptions were isolates Pa16 and Pa18, which displayed only 99% similarity with *P. aeruginosa* - GenBank accession nos. CP012901.1 (Mataseje et al., 2016) and CP025051.1 respectively. Pa16 showed a novel mutation - ^{278}T→C whilst Pa18 was found to harbour another mutation, ^{197}G→A.

Table 4.14. Summary of BLASTN analyses of *mexT* ampliconic sequences against the GenBank deposits of *P. aeruginosa*

Isolates	GenBank accession nos. showing 100% similarity with *mexT* amplicon sequence
Pa3, Pa8	CP026680.1, CP016955.1,CP008857.1
Pa4	CP028584.1, CP017306.1, CP022002.1
Pa5, Pa9, Pa17, Pa24, Pa28	CP008872.2, CP013113.1, AP014651.1
Pa7, Pa26	CP022526.1, CP022525.1, CP017099.1, AP017302.1
Pa10, Pa23	CP012901.1 (Mataseje et al., 2016)
Pa12	CP028162.1, CP027166.1, CP027171.1
Pa13, Pa21	CP008739.2 (Murugan et al. 2016)
Pa14	CP015377.1
Pa15, Pa29	CP015650.1, CP008869.2, HG974234.1 (Wright et al., 2015)
Pa20, Pa22	CP027172.1, CP021775.1, CP020704.1
Pa25, Pa27	CP027174.1, CP027165.1, CP017293.1

The *mexT* sequences from 24 *P. aeruginosa* isolates along with a set of nine other similar sequences retrieved from the public database including reference *P. aeruginosa* strain, PAO1 (GenBank accession no. AE004091.2) were subjected to Bayesian phylogram construction (Fig. 4.19). The best fit model of nucleotide sequence evolution with respect to *mexT* gene based on AIC was found to be HKY+I+G. PSRF was observed to be 1.0 and the ESS value was above 100. Phylogenetic analysis was run for 1,000,000 generations and clade credibility values were found to be >0.5. *Pseudomonas citronellolis* (GenBank accession no. CP014158.1) was taken as out group. Sequences from Pa10, Pa16 and Pa23 were found to be similar to those isolates reported from North America (GenBank accession no. CP012901.1). Pa12, Pa20 and Pa22 exhibited similarity with those from Taiwan (GenBank accession no. CP004061.1), whilst Pa13 and Pa21 were related to isolates from Tamil Nadu (GenBank accession no. CP008739.2).

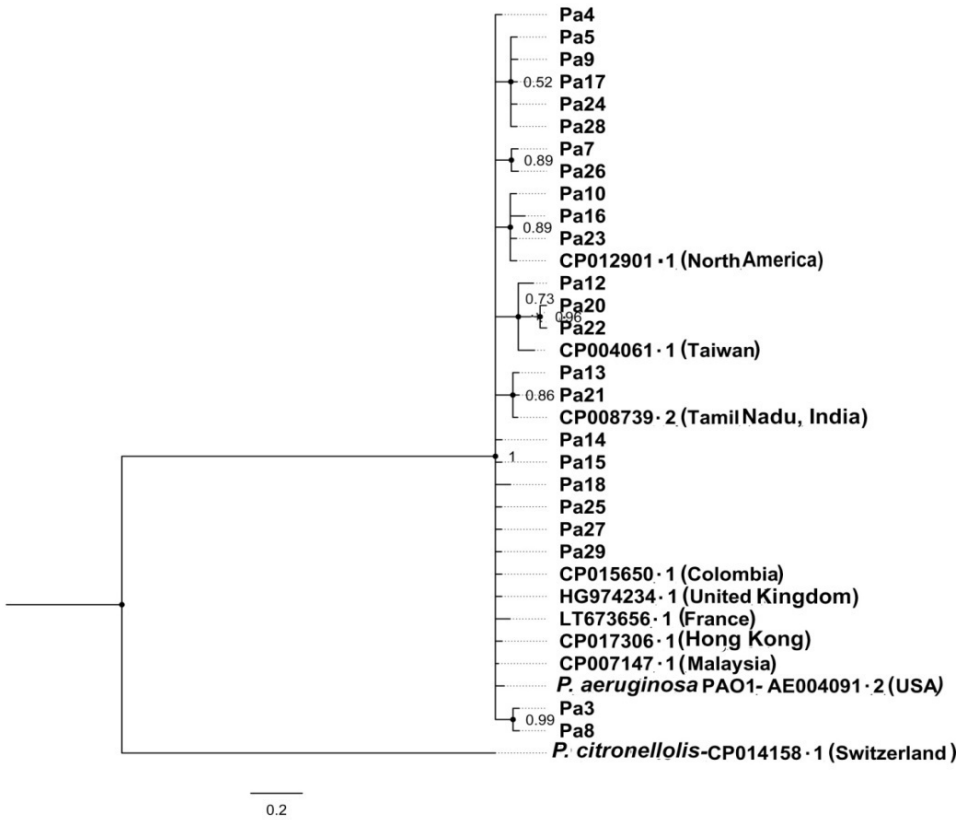


Fig. 4.19. Bayesian phylogenetic tree reconstructed employing mexT ampliconic sequences from P.aeruginosa isolates. *Scale bar denotes probability of nucleotide change. Analysis was run for 1,000,000 generations. Clade credibility values are shown at each node; P. citronellolis (GenBank accession no. CP014158.1) was used as the outgroup.*

4.4.8 Mutational variations and phylogenetic analysis of *oprD* gene

To detect mutations in the *oprD* gene encoding porin channel protein, the 1500 bp amplicons derived from the 26 isolates exhibiting porin down regulation were sequenced (Fig. 4.20). DNA sequences and the corresponding amino acids encoded were then compared with the sequences of the reference strain PAO1 (GenBank accession no. AE004091.2). On the basis of mutational patterns of the *oprD* gene, the isolates were classified into several types such as A, B1- 2, C1-4, D1-6, E1-2 and F (Table 4.15 and 4.16). An alignment of the nucleotide and the aminoacid sequences of porin genes from the isolates (OprD types) with those of the reference strain PAO1 are given in Table 4.16, wherein the various mutations are highlighted in different colours as mentioned in the footnotes. Isolates - Pa2, Pa6, Pa15 and Pa18 grouped under OprD type-A were categorized as wild type, in which both the nucleotide and amino acid sequences were found to be identical with the reference strain. Types B1 and B2 bore full length amino acid sequences except a change due to single amino acid substitution. Pa9 categorized under B1 type was found to exhibit an amino acid substitution of valine → leucine at 127^{th} position ($V_{127}L$) which showed resemblance to *P. aeruginosa* VRFPA06 isolate (GenBank accession no. ETD93478.1; Murugan et al., 2014). Pa29, a representative of B2 type, showed glycine →aspartic acid substitution at 316^{th} position ($G_{316}D$); a similar mutation was detected in *P. aeruginosa* GenBank accession no. WP_101516673.1. C1 - 4 OprD types displayed full length OprD amino acid sequences with several polymorphisms at the amino acid level. Pa4 isolate (C1 OprD type) carried 3 amino acid substitutions at 103, 115 and 170^{th} position by serine, threonine and leucine respectively (Ocampo-Sosa et al., 2012; Schiavano et al., 2017). Three isolates Pa12, Pa20 and Pa22 categorized as C2- type possessed amino acid alterations at 9 different locations of the OprD protein (Ocampo-Sosa et al., 2012; Kim et al., 2016). C3 and C4 types were found to harbour 11 substitutions with a variety of amino acids. Among these, C3 (Pa13) and C4 (Pa21) differed by amino acid changes at 2 positions – ^{299}glycine →serine ($G_{299}S$) and ^{396}glutamic acid→ glycine ($E_{396}G$) in Pa13; ^{65}tryptophan→arginine ($W_{65}R$) substitution and ^{205}lysine→glutamine ($K_{205}Q$) in Pa21. D1-6, OprD types showed a wide range of amino acid substitutions along

with nucleotide deletions and amino acid changes at 372 - 383 positions. This divergent sequence of 10 amino acid residues at 372nd (VDSSSS-YAGL-) to 383rd displayed by D types have been reported previously (Epp et al., 2001; Hammami et al., 2009; Rodríguez-Martínez et al., 2009; Ocampo-Sosa et al., 2012; Kao et al., 2016; Kim et al., 2016; Schiavano et al., 2017). Porin protein of D3 type exhibited similarities with several *P. aeruginosa* strains in the GenBank with accession nos. - ID4365 strain - KAJ07311.1 (Grosso-Becerra et al., 2014), HB15 strain - ESQ66005.1 (Soares-Castro et al., 2011) and VRFPA07 - ETD51009.1 (Murugan et al., 2014). OprD proteins of D5 type exhibited similarity to *P. aeruginosa* GenBank accession nos. KAJ11220.1 (Grosso-Becerra et al., 2014), KSC 28238.1 (Van Belkum et al., 2015) and AAS18314.2 (Edalucci et al., 2008). E1-2 types were found to exhibit several amino acid changes and presence of premature stop codons which resulted in truncated porin proteins. Pa25 isolate, representative of E1 type, carried a point mutation at the 708th nucleotide (T→G) position leading to formation of a premature stop codon, whilst premature termination was found to occur in Pa10 of E2 type by a point mutation at 1016 nucleotide position (G→A). The only isolate designated as F-type (Pa27) also displayed a premature stop codon.

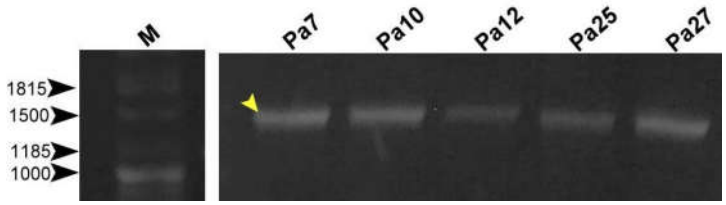


Fig. 4.20. Agarose gel (0.8%) showing oprD gene-specific amplicons from genomic-PCR of P. aeruginosa clinical isolates. *Lane denoted M represents medium range DNA ruler. Other lanes show amplicons from representatives of P. aeruginosa isolates Pa7, Pa10, Pa12,Pa25 and Pa27. Yellow arrowhead indicates 1500 bp oprD amplicon.*

Table 4.15. Different OprD types detected among *P. aeruginosa* isolates

OprD types	Isolates	Classification of OprD types	Alterations/ mutations *	Location of the structural alterations * *
A	Pa2,Pa6, Pa15, Pa18	Wild type	None	None
B1	Pa9	Full length type with single amino acid substitution	$V_{127}L$	b/w[#] L2 and L3
B2	Pa29		$G_{316}D$	L7
C1	Pa4	Full length type with several polymorphisms	$T_{103}S,K_{115}T,F_{170}L$	L2, b/w L3 and L4
C2	Pa12, Pa20, Pa22		$T_{103}S,K_{115}T,F_{170}L,E_{185}Q,$ $P_{186}G,V_{189}T,$ $R_{310}E,$ $A_{315}G,G_{425}A$	L2, b/w L3 and L4, L4,L7, L9
C3	Pa13		$T_{103}S,K_{115}T,F_{170}L,E_{185}Q,$ $P_{186}G,V_{189}T,G_{299}S,$ $R_{310}E,A_{315}G,$ $E_{396}G,G_{425}A$	L2, b/w L3 and L4, L4,L7, b/w L8 and L9, L9
C4	Pa21		$W_{65}R,T_{103}S,K_{115}T,F_{170}L,$ $E_{185}Q,P_{186}G,V_{189}T,$ $K_{205}Q,R_{310}E,A_{315}G,G_{425}A$	b/w L1 and L2, L2, b/w L3 and L4,L4, L7, L9
D1	Pa16	Several polymorphisms along with deletions and substitutions	$F_{26}L,V_{127}L,E_{185}Q,P_{186}G,$ $V_{189}T,E_{202}Q,I_{210}A,$ $E_{230}K,S_{240}T,N_{262}T,T_{276}A,$ $A_{281}G,K_{296}Q,Q_{301}E,$ $R_{310}E,$ $G_{312}R,$ $A_{315}G,L_{347}M,$ $S_{403}A,Q_{424}E$ $_{372}(VDSSSS\text{ -}YAGL\text{-})_{383}$	Prior to L1, b/w L2 and L3, L4, b/w L4 and L5, L5, b/w L5 and L6,L6, b/w L6 and L7, L7, b/wL7and L8, L8,b/w L8 and L9, L9
D2	Pa26		$S_{57}E,S_{59}R,W_{65}R,V_{127}L,$ $E_{185}Q,P_{186}G,V_{189}T,E_{202}Q,$ $I_{210}A,E_{230}K,S_{240}T,N_{262}T,$ $A_{281}G,K_{296}Q,Q_{301}E,$ $R_{310}E,A_{315}G,L_{347}M,$ $S_{403}A,W_{417}R,Q_{424}E$ $_{372}(VDSSSS\text{-}YAGL\text{-})_{383}$	L1, b/w L1 and L2, b/w L2 and L3, L4, b/w L4 and L5, L5, b/w L5 and L6, L6, b/w L6 and L7, L7, b/w L7 and L8, L8, b/w L8 and L9, L9

D3	Pa7, Pa14, Pa17, Pa24, Pa28		$S_{57}E,S_{59}R,V_{127}L,E_{185}Q,$ $P_{186}G,V_{189}T,E_{202}Q,I_{210}A,$ $E_{230}K,S_{240}T,N_{262}T,T_{276}A,$ $A_{281}G,K_{296}Q,Q_{301}E,$ $R_{310}E,A_{315}G,L_{347}M,$ $S_{403}A,Q_{424}E$ $_{372}(VDSSSS\text{-}YAGL\text{-})_{383}$	L1, b/w L2 and L3, L4, b/w L4 and L5, L5, b/w L5 and L6, L6, b/w L6 and L7, L7, b/w L7 and L8, L8, b/w L8 and L9, L9
D4	Pa8		$V_{127}L,E_{185}Q,P_{186}G,V_{189}T,$ $E_{202}Q,I_{210}A,E_{230}K,$ $S_{240}T,$ $N_{262}T,T_{276}A,A_{281}G,K_{296}Q,$ $Q_{301}E,R_{310}E,$ $G_{312}R,$ $A_{315}G,$ $W_{339}R,$ $L_{347}M,$ $S_{403}A,Q_{424}E$ $_{372}(VDSSSS\text{-}YAGL\text{-})_{383}$	b/w L2 and L3, L4, b/w L4 and L5, L5, b/w L5 and L6, L6, b/w L6 and L7, L7, b/w L7 and L8, L8, b/w L8 and L9, L9
D5	Pa5, Pa23		$V_{127}L,E_{185}Q,P_{186}G,V_{189}T,$ $E_{202}Q,I_{210}A,E_{230}K,S_{240}T,$ $N_{262}T,T_{276}A,A_{281}G,K_{296}Q,$ $Q_{301}E,$ $R_{310}E,$ $G_{312}R,$ $A_{315}G,$ $L_{347}M,$ $S_{403}A,Q_{424}E$ $_{372}(VDSSSS\text{-}YAGL\text{-})_{383}$	b/w L2 and L3, L4, b/w L4 and L5, L5, b/w L5 and L6, L6, b/w L6 and L7, L7, b/w L7 and L8, L8, b/w L8 and L9, L9
D6	Pa3		$V_{127}L,E_{185}Q,P_{186}G,V_{189}T,E_{202}$ $Q,I_{210}A,E_{230}K,S_{240}T,N_{262}T,$ $T_{276}A,A_{281}G,K_{296}Q,Q_{301}E,$ $G_{312}R,A_{315}G,L_{347}M,S_{403}A,$ $Q_{424}E$ $_{372}(VDSSSS\text{-}YAGL\text{-})_{383}$	b/w L2 and L3, L4, b/w L4 and L5, L5, b/w L5 and L6, L6, b/w L6 and L7, L7, b/w L7 and L8, L8, b/w L8 and L9, L9
E1	Pa25	Several polymorphisms and premature stop codon	$D_{43}N,S_{57}E,S_{59}R,E_{202}Q,I_{210}A,$ $E_{230}K$ $TA\underline{T}_{708}\rightarrow TA\underline{G}$ (stop codon)	Prior to L1,L4, b/w L4 and L5, L5 Truncated porin
E2	Pa10		$V_{127}L,E_{185}Q,P_{186}G,V_{189}T,$ $E_{202}Q,I_{210}A,E_{230}K,S_{240}T,$ $N_{262}T,T_{276}A,A_{281}G,K_{296}Q,$ $Q_{301}E,R_{310}E,G_{312}R,A_{315}G$ $T\underline{G}_{1016}G\rightarrow T\underline{A}G$(stop codon)	b/w L2 and L3, L4, b/w L4 and L5, L5, b/w L5 and L6, L6, b/w L6 and L7, L7 Truncated porin
F	Pa27	Premature stop codon	$_{511}\underline{G}AA\rightarrow\underline{T}AA$(stop codon)	Truncated porin

***** *Mutational alterations are denoted by single letter codes of amino acids with subscript number denoting its position in protein – the first represents the original while the second letter following the subscripted number denotes the altered one (G- Glycine, A –Alanine, L- Leucine, M- Methionine, F- Phenylalanine, W- Tryptophan, K- Lysine, Q- Glutamine, E -Glutamic Acid, S-Serine, P- Proline, V-Valine, I- Isoleucine, Y –Tyrosine, R- Arginine, N- Asparagine, D- Aspartic Acid, T –Threonine).*

****** *Loop regions (L) in the porin protein structure have been denoted according to Kos et al., 2016.*

*b/w is used here as an abbreviation for 'between'.*

Table 4.16. DNA and amino acid sequences* of *P. aeruginosa* OprD types aligned with that of the reference strain - *P. aeruginosa* PAO1

A- OprD type

Nucleotide sequence

```
A      1  ATGAAAGTGATGAAGTGGAGCGCCATTGCACTGGCGGTTTCCGCAGGTAGCACTCAGTTC  60
PAO1   1  ATGAAAGTGATGAAGTGGAGCGCCATTGCACTGGCGGTTTCCGCAGGTAGCACTCAGTTC  60

A     61  GCCGTGGCCGACGCATTCGTCAGCGATCAGGCCGAAGCGAAGGGGTTCATCGAAGACAGC  120
PAO1  61  GCCGTGGCCGACGCATTCGTCAGCGATCAGGCCGAAGCGAAGGGGTTCATCGAAGACAGC  120

A    121  AGCCTCGACCTGCTGCTCCGCAACTACTATTTCAACCGTGACGGCAAGAGCGGCAGCGGG  180
PAO1 121  AGCCTCGACCTGCTGCTCCGCAACTACTATTTCAACCGTGACGGCAAGAGCGGCAGCGGG  180

A    181  GACCGCGTCGACTGGACCCAAGGCTTCCTCACCACCTATGAATCCGGCTTCACCCAAGGC  240
PAO1 181  GACCGCGTCGACTGGACCCAAGGCTTCCTCACCACCTATGAATCCGGCTTCACCCAAGGC  240

A    241  ACTGTGGGCTTCGGCGTCGATGCCTTCGGCTACCTGGGCCTGAAGCTCGACGGCACCTCC  300
PAO1 241  ACTGTGGGCTTCGGCGTCGATGCCTTCGGCTACCTGGGCCTGAAGCTCGACGGCACCTCC  300

A    301  GACAAGACCGGCACCGGCAACCTGCCGGTGATGAACGACGGCAAGCCGCGCGATGACTAC  360
PAO1 301  GACAAGACCGGCACCGGCAACCTGCCGGTGATGAACGACGGCAAGCCGCGCGATGACTAC  360

A    361  AGCCGCGCCGGCGGCGCCGTGAAGGTGCGCATCTCCAAGACCATGCTGAAGTGGGGCGAG  420
PAO1 361  AGCCGCGCCGGCGGCGCCGTGAAGGTGCGCATCTCCAAGACCATGCTGAAGTGGGGCGAG  420

A    421  ATGCAACCGACCGCCCCGGTCTTCGCCGCTGGCGGCAGCCGCCTGTTCCCGCAGACCGCG  480
PAO1 421  ATGCAACCGACCGCCCCGGTCTTCGCCGCTGGCGGCAGCCGCCTGTTCCCGCAGACCGCG  480

A    481  ACCGGCTTCCAGCTGCAGAGCAGCGAATTCGAAGGGCTCGACCTCGAGGCAGGCCACTTC  540
PAO1 481  ACCGGCTTCCAGCTGCAGAGCAGCGAATTCGAAGGGCTCGACCTCGAGGCAGGCCACTTC  540

A    541  ACCGAGGGCAAGGAGCCGACCACCGTCAAATCGCGTGGCGAACTCTATGCCACCTACGCA  600
PAO1 541  ACCGAGGGCAAGGAGCCGACCACCGTCAAATCGCGTGGCGAACTCTATGCCACCTACGCA  600

A    601  GGCGAGACCGCCAAGAGCGCCGATTTCATTGGGGGCCGCTACGCAATCACCGATAACCTC  660
PAO1 601  GGCGAGACCGCCAAGAGCGCCGATTTCATTGGGGGCCGCTACGCAATCACCGATAACCTC  660

A    661  AGCGCCTCCCTGTACGGCGCCGAACTCGAAGACATCTATCGCCAGTATTACCTGAACAGC  720
PAO1 661  AGCGCCTCCCTGTACGGCGCCGAACTCGAAGACATCTATCGCCAGTATTACCTGAACAGC  720

A    721  AACTACACCATCCCACTGGCATCCGACCAATCGCTGGGCTTCGATTTCAACATCTACCGC  780
PAO1 721  AACTACACCATCCCACTGGCATCCGACCAATCGCTGGGCTTCGATTTCAACATCTACCGC  780

A    781  ACAAACGATGAAGGCAAGGCCAAGGCCGGCGACATCAGCAACACCACTTGGTCCCTGGCG  840
PAO1 781  ACAAACGATGAAGGCAAGGCCAAGGCCGGCGACATCAGCAACACCACTTGGTCCCTGGCG  840

A    841  GCAGCCTACACTCTGGATGCGCACACTTTCACCTTGGCCTACCAGAAGGTCCATGGCGAT  900
PAO1 841  GCAGCCTACACTCTGGATGCGCACACTTTCACCTTGGCCTACCAGAAGGTCCATGGCGAT  900

A    901  CAGCCGTTTGATTATATCGGCTTCGGCCGCAACGGCTCTGGCGCAGGTGGCGACTCGATT  960
PAO1 901  CAGCCGTTTGATTATATCGGCTTCGGCCGCAACGGCTCTGGCGCAGGTGGCGACTCGATT  960

A    961  TTCCTCGCCAACTCTGTCCAGTACTCCGACTTCAACGGCCCTGGCGAGAAATCCTGGCAG  1020
PAO1 961  TTCCTCGCCAACTCTGTCCAGTACTCCGACTTCAACGGCCCTGGCGAGAAATCCTGGCAG  1020

A    1021 GCTCGCTACGACCTGAACCTAGCCTCCTATGGCGTTCCCGGCCTGACTTTCATGGTCCGC  1080
PAO1 1021 GCTCGCTACGACCTGAACCTAGCCTCCTATGGCGTTCCCGGCCTGACTTTCATGGTCCGC  1080
```

```
A     1081TATATCAATGGCAAGGACATCGATGGCACCAAGATGTCTGACAACAACGTCGGCTATAAG 1140
PAO   1081TATATCAATGGCAAGGACATCGATGGCACCAAGATGTCTGACAACAACGTCGGCTATAAG 1140

A     1141AACTACGGCTACGGCGAGGATGGCAAGCACCACGAAACCAACCTCGAAGCCAAGTACGTG 1200
PAO1  1141AACTACGGCTACGGCGAGGATGGCAAGCACCACGAAACCAACCTCGAAGCCAAGTACGTG 1200

A     1201GTCCAGTCCGGTCCGGCCAAGGACCTGTCGTTCCGCATCCGCCAGGCCTGGCACCGTGCC 1260
PAO1  1201GTCCAGTCCGGTCCGGCCAAGGACCTGTCGTTCCGCATCCGCCAGGCCTGGCACCGTGCC 1260

A     1261AACGCCGACCAGGGCGAAGGCGACCAGAACGAGTTCCGCCTGATCGTCGACTATCCGCTG 1320
PAO1  1261AACGCCGACCAGGGCGAAGGCGACCAGAACGAGTTCCGCCTGATCGTCGACTATCCGCTG 1320

A     1321TCGATCCTGTAA  1332
PAO1  1321TCGATCCTGTAA  1332
```

Amino acid sequence

```
                                                                LOOP 1
A         MKVMKWSAIALAVSAGSTQFAVADAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSG  60
PAO1      MKVMKWSAIALAVSAGSTQFAVADAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSG  60
                                                          LOOP2
A         DRVDWTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKTGTGNLPVMNDGKPRDDY  120
PAO1      DRVDWTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKTGTGNLPVMNDGKPRDDY  120
                                  LOOP 3
A         SRAGGAVKVRISKTMLKWGEMQPTAPVFAAGGSRLFPQTATGFQLQSSEFEGLDLEAGHF  180
PAO1      SRAGGAVKVRISKTMLKWGEMQPTAPVFAAGGSRLFPQTATGFQLQSSEFEGLDLEAGHF  180

                  LOOP 4                                        LOOP 5
A         TEGKEPTTVKSRGELYATYAGETAKSADFIGGRYAITDNLSASLYGAELEDIYRQYYLNS  240
PAO1      TEGKEPTTVKSRGELYATYAGETAKSADFIGGRYAITDNLSASLYGAELEDIYRQYYLNS  240
                                  LOOP 6                          
A         NYTIPLASDQSLGFDFNIYRTNDEGKAKAGDISNTTWSLAAAYTLDAHTFTLAYQKVHGD  300
PAO1      NYTIPLASDQSLGFDFNIYRTNDEGKAKAGDISNTTWSLAAAYTLDAHTFTLAYQKVHGD  300
                       LOOP 7
A         QPFDYIGFGRNGSGAGGDSIFLANSVQYSDFNGPGEKSWQARYDLNLASYGVPGLTFMVR  360
PAO1      QPFDYIGFGRNGSGAGGDSIFLANSVQYSDFNGPGEKSWQARYDLNLASYGVPGLTFMVR  360
                            LOOP 8
A         YINGKDIDGTKMSDNNVGYKNYGYGEDGKHHETNLEAKYVVQSGPAKDLSFRIRQAWHRA  420
PAO1      YINGKDIDGTKMSDNNVGYKNYGYGEDGKHHETNLEAKYVVQSGPAKDLSFRIRQAWHRA  420
          LOOP 9
A         NADQGEGDQNEFRLIVDYPLSIL  443
PAO1      NADQGEGDQNEFRLIVDYPLSIL  443
```

B1- OprD type

Nucleotide sequence

```
B1    1     ATGAAAGTGATGAAGTGGAGCGCCATTGCACTGGCGGTTTCCGCAGGTAGCACTCAGTTC  60
PAO1  1     ATGAAAGTGATGAAGTGGAGCGCCATTGCACTGGCGGTTTCCGCAGGTAGCACTCAGTTC  60

B1    61    GCCGTGGCCGACGCATTCGTCAGCGATCAGGCCGAAGCGAAGGGGTTCATCGAAGACAGC 120
PAO1  61    GCCGTGGCCGACGCATTCGTCAGCGATCAGGCCGAAGCGAAGGGGTTCATCGAAGACAGC 120

B1    121   AGCCTCGACCTGCTGCTCCGCAACTACTATTTCAACCGTGACGGCAAGAGCGGCAGCGGG 180
 PAO1 121    AGCCTCGACCTGCTGCTCCGCAACTACTATTTCAACCGTGACGGCAAGAGCGGCAGCGGG 180

B1    181   GACCGCGTCGACTGGACCCAAGGCTTCCTCACCACCTATGAATCCGGCTTCACCCAAGGC 240
PAO1  181   GACCGCGTCGACTGGACCCAAGGCTTCCTCACCACCTATGAATCCGGCTTCACCCAAGGC 240

B1    241   ACCGTGGGCTTCGGCGTCGATGCCTTCGGCTACCTCGGCCTGAAGCTCGACGGCACCTCG 300
PAO1  241   ACTGTGGGCTTCGGCGTCGATGCCTTCGGCTACCTGGGCCTGAAGCTCGACGGCACCTCC 300
```

```
B1    301  GACAAGACCGGCACCGGCAACCTGCCGGTGATGAACGACGGCAAGCCGCGCGACGACTAC 360
PAO1  301  GACAAGACCGGCACCGGCAACCTGCCGGTGATGAACGACGGCAAGCCGCGCGATGACTAC 360

B1    361  AGCCGCGCTGGCGGCGCCCTGAAGGTGCGCATTTCCAAGACCATGCTGAAGTGGGGCGAA 420
PAO1  361  AGCCGCGCCGGCGGCGCCGTGAAGGTGCGCATCTCCAAGACCATGCTGAAGTGGGGCGAG 420

B1    421  ATGCAACCTACCGCGCCGGTCTTCGCCGCCGGCGGCAGCCGCCTGTTCCCGCAGACCGCG 480
PAO1  421  ATGCAACCGACCGCCCCGGTCTTCGCCGCTGGCGGCAGCCGCCTGTTCCCGCAGACCGCG 480

B1    481  ACCGGCTTCCAACTGCAGAGCAGTGAATTCGAAGGGCTCGATCTCGAAGCGGGCCACTTC 540
PAO1  481  ACCGGCTTCCAGCTGCAGAGCAGCGAATTCGAAGGGCTCGACCTCGAGGCAGGCCACTTC 540

B1    541  ACCGAGGGCAAGGAGCCGACCACCGTCAAATCGCGTGGCGAACTCTATGCCACCTACGCA 600
PAO1  541  ACCGAGGGCAAGGAGCCGACCACCGTCAAATCGCGTGGCGAACTCTATGCCACCTACGCA 600

B1    601  GGCGAGACCGCCAAGAGCGCCGATTTCATTGGGGGCCGCTACGCAATCACCGATAACCTC 660
PAO1  601  GGCGAGACCGCCAAGAGCGCCGATTTCATTGGGGGCCGCTACGCAATCACCGATAACCTC 660

B1    661  AGCGCCTCCCTGTACGGCGCCGAACTCGAAGACATCTATCGCCAGTATTACCTGAACAGC 720
PAO1  661  AGCGCCTCCCTGTACGGCGCCGAACTCGAAGACATCTATCGCCAGTATTACCTGAACAGC 720

B1    721  AACTACACCATCCCACTGGCATCCGACCAATCGCTGGGCTTCGATTTCAACATCTACCGC 780
PAO1  721  AACTACACCATCCCACTGGCATCCGACCAATCGCTGGGCTTCGATTTCAACATCTACCGC 780

B1    781  ACAAACGATGAAGGCAAGGCCAAGGCCGGCGACATCAGCAACACCACTTGGTCCCTGGCG 840
PAO1  781  ACAAACGATGAAGGCAAGGCCAAGGCCGGCGACATCAGCAACACCACTTGGTCCCTGGCG 840

B1    841  GCAGCCTACACTCTGGATGCGCACACTTTCACCTTGGCCTACCAGAAGGTCCATGGCGAT 900
PAO1  841  GCAGCCTACACTCTGGATGCGCACACTTTCACCTTGGCCTACCAGAAGGTCCATGGCGAT 900

B1    901  CAGCCGTTTGATTATATCGGCTTCGGCCGCAACGGCTCTGGCGCAGGTGGCGACTCGATT 960
PAO1  901  CAGCCGTTTGATTATATCGGCTTCGGCCGCAACGGCTCTGGCGCAGGTGGCGACTCGATT 960

B1    961  TTCCTCGCCAACTCTGTCCAGTACTCCGACTTCAACGGCCCTGGCGAGAAATCCTGGCAG 1020
PAO1  961  TTCCTCGCCAACTCTGTCCAGTACTCCGACTTCAACGGCCCTGGCGAGAAATCCTGGCAG 1020

B1   1021  GCTCGCTACGACCTGAACCTAGCCTCCTATGGCGTTCCCGGCCTGACTTTCATGGTCCGC 1080
PAO1 1021  GCTCGCTACGACCTGAACCTAGCCTCCTATGGCGTTCCCGGCCTGACTTTCATGGTCCGC 1080

B1   1081  TATATCAATGGCAAGGACATCGATGGCACCAAGATGTCTGACAACAACGTCGGCTATAAG 1140
PAO  1081  TATATCAATGGCAAGGACATCGATGGCACCAAGATGTCTGACAACAACGTCGGCTATAAG 1140

B1   1141  AACTACGGCTACGGCGAGGACGGCAAGCACCACGAGACCAACCTCGAAGCCAAGTACGTG 1200
PAO1 1141  AACTACGGCTACGGCGAGGATGGCAAGCACCACGAAACCAACCTCGAAGCCAAGTACGTG 1200

B1   1201  GTCCAGTCCGGTCCGGCCAAGGACCTGTCGTTCCGCATCCGCCAGGCCTGGCACCGTGCC 1260
PAO1 1201  GTCCAGTCCGGTCCGGCCAAGGACCTGTCGTTCCGCATCCGCCAGGCCTGGCACCGTGCC 1260

B1   1261  AACGCCGACCAGGGCGAAGGCGACCAGAACGAGTTCCGCCTGATCGTCGACTATCCGCTG 1320
PAO1 1261  AACGCCGACCAGGGCGAAGGCGACCAGAACGAGTTCCGCCTGATCGTCGACTATCCGCTG 1320

B1   1321      TCGATCCTGTAA  1332
PAO1 1321      TCGATCCTGTAA  1332
```

Amino acid sequence

```
B1       MKVMKWSAIALAVSAGSTQFAVADAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSG   60
PAO1     MKVMKWSAIALAVSAGSTQFAVADAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSG   60

B1       DRVDWTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKTGTGNLPVMNDGKPRDDY   120
PAO1     DRVDWTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKTGTGNLPVMNDGKPRDDY   120

B1       SRAGGALKVRISKTMLKWGEMQPTAPVFAAGGSRLFPQTATGFQLQSSEFEGLDLEAGHF   180
PAO1     SRAGGAVKVRISKTMLKWGEMQPTAPVFAAGGSRLFPQTATGFQLQSSEFEGLDLEAGHF   180
```

```
B1       TEGKEPTTVKSRGELYATYAGETAKSADFIGGRYAITDNLSASLYGAELEDIYRQYYLNS   240
PAO1     TEGKEPTTVKSRGELYATYAGETAKSADFIGGRYAITDNLSASLYGAELEDIYRQYYLNS   240

B1      NYTIPLASDQSLGFDFNIYRTNDEGKAKAGDISNTTWSLAAAYTLDAHTFTLAYQKVHGD    300
PAO1    NYTIPLASDQSLGFDFNIYRTNDEGKAKAGDISNTTWSLAAAYTLDAHTFTLAYQKVHGD    300

B1      QPFDYIGFGRNGSGAGGDSIFLANSVQYSDFNGPGEKSWQARYDLNLASYGVPGLTFMVR    360
PAO1    QPFDYIGFGRNGSGAGGDSIFLANSVQYSDFNGPGEKSWQARYDLNLASYGVPGLTFMVR    360

B1      YINGKDIDGTKMSDNNVGYKNYGYGEDGKHHETNLEAKYVVQSGPAKDLSFRIRQAWHRA    420
PAO1    YINGKDIDGTKMSDNNVGYKNYGYGEDGKHHETNLEAKYVVQSGPAKDLSFRIRQAWHRA    420

B1     NADQGEGDQNEFRLIVDYPLSIL  443
PAO1   NADQGEGDQNEFRLIVDYPLSIL  443
```

B2- OprD type

Nucleotide sequence

```
B2     1  ATGAAAGTGATGAAGTGGAGCGCCATTGCACTGGCGGTTTCCGCAGGTAGCACTCAGTTC   60
PAO1   1  ATGAAAGTGATGAAGTGGAGCGCCATTGCACTGGCGGTTTCCGCAGGTAGCACTCAGTTC   60

B2    61  GCCGTGGCCGACGCATTCGTCAGCGATCAGGCCGAAGCGAAGGGGTTCATCGAAGACAGC   120
PAO1  61  GCCGTGGCCGACGCATTCGTCAGCGATCAGGCCGAAGCGAAGGGGTTCATCGAAGACAGC   120

B2   121  AGCCTCGACCTGCTGCTCCGCAACTACTATTTCAACCGTGACGGCAAGAGCGGCAGCGGG   180
PAO1 121  AGCCTCGACCTGCTGCTCCGCAACTACTATTTCAACCGTGACGGCAAGAGCGGCAGCGGG   180

B2   181  GACCGCGTCGACTGGACCCAAGGCTTCCTCACCACCTATGAATCCGGCTTCACCCAAGGC   240
PAO1 181  GACCGCGTCGACTGGACCCAAGGCTTCCTCACCACCTATGAATCCGGCTTCACCCAAGGC   240

B2   241  ACTGTGGGCTTCGGCGTCGATGCCTTCGGCTACCTGGGCCTGAAGCTCGACGGCACCTCC   300
PAO1 241  ACTGTGGGCTTCGGCGTCGATGCCTTCGGCTACCTGGGCCTGAAGCTCGACGGCACCTCC   300

B2   301  GACAAGACCGGCACCGGCAACCTGCCGGTGATGAACGACGGCAAGCCGCGCGATGACTAC   360
PAO1 301  GACAAGACCGGCACCGGCAACCTGCCGGTGATGAACGACGGCAAGCCGCGCGATGACTAC   360

B2   361  AGCCGCGCCGGCGGCGCCGTGAAGGTGCGCATCTCCAAGACCATGCTGAAGTGGGGCGAG   420
PAO1 361  AGCCGCGCCGGCGGCGCCGTGAAGGTGCGCATCTCCAAGACCATGCTGAAGTGGGGCGAG   420

B2   421  ATGCAACCGACCGCCCCGGTCTTCGCCGCTGGCGGCAGCCGCCTGTTCCCGCAGACCGCG   480
PAO1 421  ATGCAACCGACCGCCCCGGTCTTCGCCGCTGGCGGCAGCCGCCTGTTCCCGCAGACCGCG   480

B2   481  ACCGGCTTCCAGCTGCAGAGCAGCGAATTCGAAGGGCTCGACCTCGAGGCAGGCCACTTC   540
PAO1 481  ACCGGCTTCCAGCTGCAGAGCAGCGAATTCGAAGGGCTCGACCTCGAGGCAGGCCACTTC   540

B2   541  ACCGAGGGCAAGGAGCCGACCACCGTCAAATCGCGTGGCGAACTCTATGCCACCTACGCA   600
PAO1 541  ACCGAGGGCAAGGAGCCGACCACCGTCAAATCGCGTGGCGAACTCTATGCCACCTACGCA   600

B2   601  GGCGAGACCGCCAAGAGCGCCGATTTCATTGGGGGCCGCTACGCAATCACCGATAACCTC   660
PAO1 601  GGCGAGACCGCCAAGAGCGCCGATTTCATTGGGGGCCGCTACGCAATCACCGATAACCTC   660

B2   661  AGCGCCTCCCTGTACGGCGCCGAACTCGAAGACATCTATCGCCAGTATTACCTGAACAGC   720
PAO1 661  AGCGCCTCCCTGTACGGCGCCGAACTCGAAGACATCTATCGCCAGTATTACCTGAACAGC   720

B2   721  AACTACACCATCCCACTGGCATCCGACCAATCGCTGGGCTTCGATTTCAACATCTACCGC   780
PAO1 721  AACTACACCATCCCACTGGCATCCGACCAATCGCTGGGCTTCGATTTCAACATCTACCGC   780

B2   781  ACAAACGATGAAGGCAAGGCCAAGGCCGGCGACATCAGCAACACCACTTGGTCCCTGGCG   840
PAO1 781  ACAAACGATGAAGGCAAGGCCAAGGCCGGCGACATCAGCAACACCACTTGGTCCCTGGCG   840

B2   841  GCAGCCTACACTCTGGATGCGCACACTTTCACCTTGGCCTACCAGAAGGTCCATGGCGAT   900
PAO1 841  GCAGCCTACACTCTGGATGCGCACACTTTCACCTTGGCCTACCAGAAGGTCCATGGCGAT   900
```

```
B2    901 CAGCCGTTTGATTATATCGGCTTCGGCCGCAACGGCTCTGGCGCAGATGGCGACTCGATT  960
PAO1  901 CAGCCGTTTGATTATATCGGCTTCGGCCGCAACGGCTCTGGCGCAGGTGGCGACTCGATT  960

B2    961 TTCCTCGCCAACTCTGTCCAGTACTCCGACTTCAACGGCCCTGGCGAGAAATCCTGGCAG  1020
PAO1  961 TTCCTCGCCAACTCTGTCCAGTACTCCGACTTCAACGGCCCTGGCGAGAAATCCTGGCAG  1020

B2    1021 GCTCGCTACGACCTGAACCTAGCCTCCTATGGCGTTCCCGGCCTGACTTTCATGGTCCGC 1080
PAO1  1021 GCTCGCTACGACCTGAACCTAGCCTCCTATGGCGTTCCCGGCCTGACTTTCATGGTCCGC 1080

B2    1081 TATATCAATGGCAAGGACATCGATGGCACCAAGATGTCTGACAACAACGTCGGCTATAAG 1140
PAO1  1081 TATATCAATGGCAAGGACATCGATGGCACCAAGATGTCTGACAACAACGTCGGCTATAAG 1140

B2    1141 AACTACGGCTACGGCGAGGATGGCAAGCACCACGAAACCAACCTCGAAGCCAAGTACGTG 1200
PAO1  1141 AACTACGGCTACGGCGAGGATGGCAAGCACCACGAAACCAACCTCGAAGCCAAGTACGTG 1200

B2    1201 GTCCAGTCCGGTCCGGCCAAGGACCTGTCGTTCCGCATCCGCCAGGCCTGGCACCGTGCC 1260
PAO1  1201 GTCCAGTCCGGTCCGGCCAAGGACCTGTCGTTCCGCATCCGCCAGGCCTGGCACCGTGCC 1260

B2    1261 AACGCCGACCAGGGCGAAGGCGACCAGAACGAGTTCCGCCTGATCGTCGACTATCCGCTG 1320
PAO1  1261 AACGCCGACCAGGGCGAAGGCGACCAGAACGAGTTCCGCCTGATCGTCGACTATCCGCTG 1320

B2    1321 TCGATCCTGTAA  1332
PAO1  1321 TCGATCCTGTAA  1332
```

Amino acid sequence

```
B2      MKVMKWSAIALAVSAGSTQFAVADAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSG    60
PAO1    MKVMKWSAIALAVSAGSTQFAVADAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSG    60

B2      DRVDWTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKTGTGNLPVMNDGKPRDDY    120
PAO1    DRVDWTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKTGTGNLPVMNDGKPRDDY    120

B2      SRAGGAVKVRISKTMLKWGEMQPTAPVFAAGGSRLFPQTATGFQLQSSEFEGLDLEAGHF    180
PAO1    SRAGGAVKVRISKTMLKWGEMQPTAPVFAAGGSRLFPQTATGFQLQSSEFEGLDLEAGHF    180

B2      TEGKEPTTVKSRGELYATYAGETAKSADFIGGRYAITDNLSASLYGAELEDIYRQYYLNS    240
PAO1    TEGKEPTTVKSRGELYATYAGETAKSADFIGGRYAITDNLSASLYGAELEDIYRQYYLNS    240

B2      NYTIPLASDQSLGFDFNIYRTNDEGKAKAGDISNTTWSLAAAYTLDAHTFTLAYQKVHGD    300
PAO1    NYTIPLASDQSLGFDFNIYRTNDEGKAKAGDISNTTWSLAAAYTLDAHTFTLAYQKVHGD    300

B2      QPFDYIGFGRNGSGADGDSIFLANSVQYSDFNGPGEKSWQARYDLNLASYGVPGLTFMVR    360
PAO1    QPFDYIGFGRNGSGAGGDSIFLANSVQYSDFNGPGEKSWQARYDLNLASYGVPGLTFMVR    360

B2      YINGKDIDGTKMSDNNVGYKNYGYGEDGKHHETNLEAKYVVQSGPAKDLSFRIRQAWHRA    420
PAO1    YINGKDIDGTKMSDNNVGYKNYGYGEDGKHHETNLEAKYVVQSGPAKDLSFRIRQAWHRA    420

B2      NADQGEGDQNEFRLIVDYPLSIL  443
PAO1    NADQGEGDQNEFRLIVDYPLSIL  443
```

C1- OprD type

Nucleotide sequence

```
C1    1   ATGAAAGTGATGAAGTGGAGCGCCATTGCACTGGCGGTTTCCGCAGGTAGCACTCAGTTC   60
PAO1  1   ATGAAAGTGATGAAGTGGAGCGCCATTGCACTGGCGGTTTCCGCAGGTAGCACTCAGTTC   60

C1    61  GCCGTGGCCGACGCATTCGTCAGCGATCAGGCCGAAGCGAAGGGGTTCATCGAAGACAGC   120
PAO1  61  GCCGTGGCCGACGCATTCGTCAGCGATCAGGCCGAAGCGAAGGGGTTCATCGAAGACAGC   120
```

```
C1    121  AGCCTCGACCTGCTGCTCCGCAACTACTATTTCAACCGTGACGGCAAGAGCGGCAGCGGG  180
PAO1  121  AGCCTCGACCTGCTGCTCCGCAACTACTATTTCAACCGTGACGGCAAGAGCGGCAGCGGG  180

C1    181  GACCGCGTCGACTGGACCCAAGGCTTCCTCACCACCTATGAATCCGGCTTCACCCAAGGC  240
PAO1  181  GACCGCGTCGACTGGACCCAAGGCTTCCTCACCACCTATGAATCCGGCTTCACCCAAGGC  240

C1    241  ACTGTGGGCTTCGGCGTCGATGCCTTCGGCTACCTCGGCCTGAAGCTCGACGGTACCTCT  300
PAO1  241  ACTGTGGGCTTCGGCGTCGATGCCTTCGGCTACCTGGGCCTGAAGCTCGACGGCACCTCC  300

C1    301  GACAAGAGCGGCACCGGCAACCTGCCAGTAATGAACGACGGAACGCCCCGTGACGACTAC  360
PAO1  301  GACAAGACCGGCACCGGCAACCTGCCGGTGATGAACGACGGCAAGCCGCGCGATGACTAC  360

C1    361  AGCCGCGCCGGTGGCGCCGTGAAGGTACGCATCTCCAAGACCATGCTGAAGTGGGGCGAG  420
PAO1  361  AGCCGCGCCGGCGGCGCCGTGAAGGTGCGCATCTCCAAGACCATGCTGAAGTGGGGCGAG  420

C1    421  ATGCAGCCGACCGCTCCGGTCTTCGCCGCTGGGGGCAGCCGCCTGTTCCCCCAGACCGCG  480
PAO1  421  ATGCAACCGACCGCCCCGGTCTTCGCCGCTGGCGGCAGCCGCCTGTTCCCGCAGACCGCG  480

C1    481  ACCGGCTTCCAGCTGCAGAGCAGCGAACTCGAAGGGCTCGACCTCGAGGCAGGCCACTTC  540
PAO1  481  ACCGGCTTCCAGCTGCAGAGCAGCGAATTCGAAGGGCTCGACCTCGAGGCAGGCCACTTC  540

C1    541  ACCGAGGGCAAGGAGCCGACCACCGTCAAATCGCGTGGCGAACTCTATGCCACCTACGCA  600
PAO1  541  ACCGAGGGCAAGGAGCCGACCACCGTCAAATCGCGTGGCGAACTCTATGCCACCTACGCA  600

C1    601  GGCGAGACCGCCAAGAGCGCCGATTTCATTGGGGGCCGCTACGCAATCACCGATAACCTC  660
PAO1  601  GGCGAGACCGCCAAGAGCGCCGATTTCATTGGGGGCCGCTACGCAATCACCGATAACCTC  660


C1    661  AGCGCCTCCCTGTACGGCGCCGAACTCGAAGACATCTATCGCCAGTATTACCTGAACAGC  720
PAO1  661  AGCGCCTCCCTGTACGGCGCCGAACTCGAAGACATCTATCGCCAGTATTACCTGAACAGC  720

C1    721  AACTACACCATCCCACTGGCATCCGACCAATCGCTGGGCTTCGATTTCAACATCTACCGC  780
PAO1  721  AACTACACCATCCCACTGGCATCCGACCAATCGCTGGGCTTCGATTTCAACATCTACCGC  780

C1    781  ACAAACGATGAAGGCAAGGCCAAGGCCGGCGACATCAGCAACACCACTTGGTCCCTGGCG  840
PAO1  781  ACAAACGATGAAGGCAAGGCCAAGGCCGGCGACATCAGCAACACCACTTGGTCCCTGGCG  840

C1    841  GCAGCCTACACTCTGGATGCGCACACTTTCACCTTGGCCTACCAGAAGGTCCATGGCGAT  900
PAO1  841  GCAGCCTACACTCTGGATGCGCACACTTTCACCTTGGCCTACCAGAAGGTCCATGGCGAT  900

C1    901  CAGCCGTTTGATTATATCGGCTTCGGCCGCAACGGCTCTGGCGCAGGTGGCGACTCGATT  960
PAO1  901  CAGCCGTTTGATTATATCGGCTTCGGCCGCAACGGCTCTGGCGCAGGTGGCGACTCGATT  960

C1    961  TTCCTCGCCAACTCTGTCCAGTACTCCGACTTCAACGGCCCTGGCGAGAAATCCTGGCAG 1020
PAO1  961  TTCCTCGCCAACTCTGTCCAGTACTCCGACTTCAACGGCCCTGGCGAGAAATCCTGGCAG 1020

C1   1021  GCTCGCTACGACCTGAACCTAGCCTCCTATGGCGTTCCCGGCCTGACTTTCATGGTCCGC 1080
PAO1 1021  GCTCGCTACGACCTGAACCTAGCCTCCTATGGCGTTCCCGGCCTGACTTTCATGGTCCGC 1080

C1   1081  TATATCAATGGCAAGGACATCGATGGCACCAAGATGTCTGACAACAACGTCGGCTATAAG 1140
PAO1 1081  TATATCAATGGCAAGGACATCGATGGCACCAAGATGTCTGACAACAACGTCGGCTATAAG 1140

C1   1141  AACTACGGCTACGGCGAGGACGGCAAGCACCACGAGACCAACCTCGAAGCCAAGTACGTG 1200
PAO1 1141  AACTACGGCTACGGCGAGGATGGCAAGCACCACGAAACCAACCTCGAAGCCAAGTACGTG 1200

C1   1201  GTCCAGTCCGGTCCGGCCAAGGACCTGTCGTTCCGCATCCGCCAGGCCTGGCACCGTGCC 1260
PAO1 1201  GTCCAGTCCGGTCCGGCCAAGGACCTGTCGTTCCGCATCCGCCAGGCCTGGCACCGTGCC 1260

C1   1261  AACGCCGACCAGGGCGAAGGCGACCAGAACGAGTTCCGCCTGATCGTCGACTATCCGCTG 1320
PAO1 1261  AACGCCGACCAGGGCGAAGGCGACCAGAACGAGTTCCGCCTGATCGTCGACTATCCGCTG 1320

C1   1321  TCGATCCTGTAA  1332
PAO1 1321  TCGATCCTGTAA  1332
```

Amino acid sequence

```
C1     MKVMKWSAIALAVSAGSTQFAVADAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSG    60
PAO1   MKVMKWSAIALAVSAGSTQFAVADAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSG    60

C1     DRVDWTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKSGTGNLPVMNDGTPRDDY    120
PAO1   DRVDWTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKTGTGNLPVMNDGKPRDDY    120

C1     SRAGGAVKVRISKTMLKWGEMQPTAPVFAAGGSRLFPQTATGFQLQSSELEGLDLEAGHF    180
PAO1   SRAGGAVKVRISKTMLKWGEMQPTAPVFAAGGSRLFPQTATGFQLQSSEFEGLDLEAGHF    180

C1     TEGKEPTTVKSRGELYATYAGETAKSADFIGGRYAITDNLSASLYGAELEDIYRQYYLNS    240
PAO1   TEGKEPTTVKSRGELYATYAGETAKSADFIGGRYAITDNLSASLYGAELEDIYRQYYLNS    240

C1     NYTIPLASDQSLGFDFNIYRTNDEGKAKAGDISNTTWSLAAAYTLDAHTFTLAYQKVHGD    300
PAO1   NYTIPLASDQSLGFDFNIYRTNDEGKAKAGDISNTTWSLAAAYTLDAHTFTLAYQKVHGD    300

C1     QPFDYIGFGRNGSGAGGDSIFLANSVQYSDFNGPGEKSWQARYDLNLASYGVPGLTFMVR    360
PAO1   QPFDYIGFGRNGSGAGGDSIFLANSVQYSDFNGPGEKSWQARYDLNLASYGVPGLTFMVR    360

C1     YINGKDIDGTKMSDNNVGYKNYGYGEDGKHHETNLEAKYVVQSGPAKDLSFRIRQAWHRA    420
PAO1   YINGKDIDGTKMSDNNVGYKNYGYGEDGKHHETNLEAKYVVQSGPAKDLSFRIRQAWHRA    420

C1     NADQGEGDQNEFRLIVDYPLSIL  443
PAO1   NADQGEGDQNEFRLIVDYPLSIL  443
```

C2- OprD type

Nucleotide sequence

```
C2     1   ATGAAAGTGATGAAGTGGAGCGCCATTGCACTGGCGGTTTCCGCAGGTAGCACTCAGTTC  60
PAO1   1   ATGAAAGTGATGAAGTGGAGCGCCATTGCACTGGCGGTTTCCGCAGGTAGCACTCAGTTC  60

C2     61  GCCGTGGCCGACGCATTCGTCAGCGATCAAGCCGAAGCGAAGGGGTTCATCGAAGACAGC  120
PAO1   61  GCCGTGGCCGACGCATTCGTCAGCGATCAGGCCGAAGCGAAGGGGTTCATCGAAGACAGC  120

C2     121 AGCCTCGACCTGCTGCTCCGCAACTACTATTTCAACCGTGACGGCAAGAGCGGCAGCGGG  180
PAO1   121 AGCCTCGACCTGCTGCTCCGCAACTACTATTTCAACCGTGACGGCAAGAGCGGCAGCGGG  180

C2     181 GACCGCGTCGACTGGACCCAAGGCTTCCTCACCACCTATGAATCCGGCTTCACCCAAGGC  240
PAO1   181 GACCGCGTCGACTGGACCCAAGGCTTCCTCACCACCTATGAATCCGGCTTCACCCAAGGC  240

C2     241 ACCGTCGGCTTCGGCGTCGATGCCTTCGGCTACCTCGGTCTGAAGCTCGACGGCACCTCC  300
PAO1   241 ACTGTGGGCTTCGGCGTCGATGCCTTCGGCTACCTGGGCCTGAAGCTCGACGGCACCTCC  300

C2     301 GACAAGAGTGGTACCGGCAACCTGCCAGTGATGAACGACGGCACGCCCCGTGACGACTAC  360
PAO1   301 GACAAGACCGGCACCGGCAACCTGCCGGTGATGAACGACGGCAAGCCGCGCGATGACTAC  360

C2     361 AGCCGCGCCGGTGGCGCCGTGAAGGTACGCATCTCCAAGACCATGTTGAAGTGGGGCGAG  420
PAO1   361 AGCCGCGCCGGCGGCGCCGTGAAGGTGCGCATCTCCAAGACCATGCTGAAGTGGGGCGAG  420

C2     421 ATGCAGCCGACCGCTCCGGTCTTCGCCGCCGGCGGCAGCCGCCTGTTCCCGCAGACCGCG  480
PAO1   421 ATGCAACCGACCGCCCCGGTCTTCGCCGCTGGCGGCAGCCGCCTGTTCCCGCAGACCGCG  480

C2     481 ACCGGCTTCCAACTGCAGAGCAGCGAACTCGAAGGGCTCGATCTCGAAGCGGGCCACTTC  540
PAO1   481 ACCGGCTTCCAGCTGCAGAGCAGCGAATTCGAAGGGCTCGACCTCGAGGCAGGCCACTTC  540

C2     541 ACCGAAGGCAAGCAGGGCACCACCACCAAGTCGCGCGGCGAACTCTACGCAACCTATGCA  600
PAO1   541 ACCGAGGGCAAGGAGCCGACCACCGTCAAATCGCGTGGCGAACTCTATGCCACCTACGCA  600

C2     601 GGCGAGACCGCCAAGAGCGCCGATTTCATTGGGGGCCGCTACGCAATCACCGATAACCTC  660
PAO1   601 GGCGAGACCGCCAAGAGCGCCGATTTCATTGGGGGCCGCTACGCAATCACCGATAACCTC  660
```

```
C2    661  AGCGCCTCCCTGTACGGTGCTGAACTCGAAGACATCTATCGTCAGTATTACCTGAACAGC  720
PAO1  661  AGCGCCTCCCTGTACGGCGCCGAACTCGAAGACATCTATCGCCAGTATTACCTGAACAGC  720

C2    721  AACTACACCATCCCACTGGCATCCGACCAATCGCTGGGCTTCGATTTCAACATCTACCGC  780
PAO1  721  AACTACACCATCCCACTGGCATCCGACCAATCGCTGGGCTTCGATTTCAACATCTACCGC  780

C2    781  ACAAACGATGAAGGCAAGGCCAAGGCCGGCGACATCAGCAACACCACTTGGTCCCTGGCG  840
PAO1  781  ACAAACGATGAAGGCAAGGCCAAGGCCGGCGACATCAGCAACACCACTTGGTCCCTGGCG  840

C2    841  GCAGCCTACACTCTGGATGCGCACACTTTCACCTTGGCCTACCAGAAGGTCCATGGCGAT  900
PAO1  841  GCAGCCTACACTCTGGATGCGCACACTTTCACCTTGGCCTACCAGAAGGTCCATGGCGAT  900

C2    901  CAGCCGTTTGATTATATCGGCTTCGGCGAGAACGGTTCCGGCGGCGGCGGTGACTCGATT  960
PAO1  901  CAGCCGTTTGATTATATCGGCTTCGGCCGCAACGGCTCTGGCGCAGGTGGCGACTCGATT  960

C2    961  TTCCTCGCCAACTCCGTGCAGTACTCCGACTTCAACGGCCCCGGCGAGAAATCCTGGCAG 1020
PAO1  961  TTCCTCGCCAACTCTGTCCAGTACTCCGACTTCAACGGCCCTGGCGAGAAATCCTGGCAG 1020

C2   1021  GCCCGCTACGACCTGAACCTCGCCTCCTATGGCGTTCCCGGCCTGACTTTCATGGTCCGC 1080
PAO1 1021  GCTCGCTACGACCTGAACCTAGCCTCCTATGGCGTTCCCGGCCTGACTTTCATGGTCCGC 1080

C2   1081  TATATCAATGGCAAGGACATCGATGGCACCAAGATGTCTGACAACAACGTCGGCTATAAG 1140
PAO1 1081  TATATCAATGGCAAGGACATCGATGGCACCAAGATGTCTGACAACAACGTCGGCTATAAG 1140

C2   1141  AACTACGGCTACGGCGAGGACGGCAAGCACCACGAGACCAACCTGGAAGCCAAGTACGTG 1200
PAO1 1141  AACTACGGCTACGGCGAGGATGGCAAGCACCACGAAACCAACCTCGAAGCCAAGTACGTG 1200

C2   1201  GTCCAGTCCGGTCCGGCCAAGGACCTGTCGTTCCGCATCCGCCAGGCCTGGCACCGCGCC 1260
PAO1 1201  GTCCAGTCCGGTCCGGCCAAGGACCTGTCGTTCCGCATCCGCCAGGCCTGGCACCGTGCC 1260

C2   1261  AACGCCGACCAGGCCGAAGGCGACCAGAACGAGTTCCGCCTGATCGTCGACTATCCGCTG 1320
PAO1 1261  AACGCCGACCAGGGCGAAGGCGACCAGAACGAGTTCCGCCTGATCGTCGACTATCCGCTG 1320

C2   1321  TCGATCCTGTAA  1332
PAO1 1321  TCGATCCTGTAA  1332
```

Amino acid sequence

```
C2      MKVMKWSAIALAVSAGSTQFAVADAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSG   60
PAO1    MKVMKWSAIALAVSAGSTQFAVADAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSG   60

C2      DRVDWTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKSGTGNLPVMNDGTPRDDY  120
PAO1    DRVDWTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKTGTGNLPVMNDGKPRDDY  120

C2      SRAGGAVKVRISKTMLKWGEMQPTAPVFAAGGSRLFPQTATGFQLQSSELEGLDLEAGHF  180
PAO1    SRAGGAVKVRISKTMLKWGEMQPTAPVFAAGGSRLFPQTATGFQLQSSEFEGLDLEAGHF  180

C2      TEGKQGTTTKSRGELYATYAGETAKSADFIGGRYAITDNLSASLYGAELEDIYRQYYLNS  240
PAO1    TEGKEPTTVKSRGELYATYAGETAKSADFIGGRYAITDNLSASLYGAELEDIYRQYYLNS  240

C2      NYTIPLASDQSLGFDFNIYRTNDEGKAKAGDISNTTWSLAAAYTLDAHTFTLAYQKVHGD  300
PAO1    NYTIPLASDQSLGFDFNIYRTNDEGKAKAGDISNTTWSLAAAYTLDAHTFTLAYQKVHGD  300

C2      QPFDYIGFGENGSGGGGDSIFLANSVQYSDFNGPGEKSWQARYDLNLASYGVPGLTFMVR  360
PAO1    QPFDYIGFGRNGSGAGGDSIFLANSVQYSDFNGPGEKSWQARYDLNLASYGVPGLTFMVR  360

C2      YINGKDIDGTKMSDNNVGYKNYGYGEDGKHHETNLEAKYVVQSGPAKDLSFRIRQAWHRA  420
PAO1    YINGKDIDGTKMSDNNVGYKNYGYGEDGKHHETNLEAKYVVQSGPAKDLSFRIRQAWHRA  420

C2      NADQAEGDQNEFRLIVDYPLSIL  443
PAO1    NADQGEGDQNEFRLIVDYPLSIL  443
```

C3- OprD type

Nucleotide sequence

```
C3      1  ATGAAAGTGATGAAGTGGAGCGCCATTGCACTGGCGGTTTCCGCAGGTAGCACTCAGTTC  60
PAO1    1  ATGAAAGTGATGAAGTGGAGCGCCATTGCACTGGCGGTTTCCGCAGGTAGCACTCAGTTC  60

C3     61  GCCGTAGCCGACGCATTCGTCAGCGATCAAGCCGAAGCGAAGGGGTTCATCGAAGACAGC  120
PAO1   61  GCCGTGGCCGACGCATTCGTCAGCGATCAGGCCGAAGCGAAGGGGTTCATCGAAGACAGC  120

C3    121  AGCCTCGACCTGCTGCTCCGCAACTACTATTTCAACCGTGACGGCAAGAGCGGCAGCGGG  180
PAO1  121  AGCCTCGACCTGCTGCTCCGCAACTACTATTTCAACCGTGACGGCAAGAGCGGCAGCGGG  180

C3    181  GACCGCGTCGACTGGACCCAAGGCTTCCTCACCACCTATGAATCCGGCTTCACCCAAGGC  240
PAO1  181  GACCGCGTCGACTGGACCCAAGGCTTCCTCACCACCTATGAATCCGGCTTCACCCAAGGC  240

C3    241  ACCGTCGGCTTCGGCGTCGATGCCTTCGGCTACCTCGGTCTGAAGCTCGACGGCACCTCC  300
PAO1  241  ACTGTGGGCTTCGGCGTCGATGCCTTCGGCTACCTGGGCCTGAAGCTCGACGGCACCTCC  300

C3    301  GACAAGAGCGGTACCGGCAACCTGCCAGTGATGAACGACGGCACGCCCCGTGACGACTAC  360
PAO1  301  GACAAGACCGGCACCGGCAACCTGCCGGTGATGAACGACGGCAAGCCGCGCGATGACTAC  360

C3    361  AGCCGCGCCGGTGGCGCCGTGAAGGTACGCATCTCCAAGACCATGTTGAAGTGGGGCGAG  420
PAO1  361  AGCCGCGCCGGCGGCGCCGTGAAGGTGCGCATCTCCAAGACCATGCTGAAGTGGGGCGAG  420

C3    421  ATGCAGCCGACCGCTCCGGTCTTCGCCGCCGGCGGCAGCCGCCTGTTCCCGCAGACCGCG  480
PAO1  421  ATGCAACCGACCGCCCCGGTCTTCGCCGCTGGCGGCAGCCGCCTGTTCCCGCAGACCGCG  480

C3    481  ACCGGCTTCCAATTGCAGAGCAGCGAACTCGAAGGGCTCGATCTCGAAGCGGGCCACTTC  540
PAO1  481  ACCGGCTTCCAGCTGCAGAGCAGCGAATTCGAAGGGCTCGACCTCGAGGCAGGCCACTTC  540

C3    541  ACCGAAGGCAAGCAGGGCACCACCACCAAGTCGCGCGGCGAACTCTACGCAACCTATGCA  600
PAO1  541  ACCGAGGGCAAGGAGCCGACCACCGTCAAATCGCGTGGCGAACTCTATGCCACCTACGCA  600

C3    601  GGCGAGACCGCCAAGAGCGCCGATTTCATTGGGGGCCGCTACGCAATCACCGATAACCTC  660
PAO1  601  GGCGAGACCGCCAAGAGCGCCGATTTCATTGGGGGCCGCTACGCAATCACCGATAACCTC  660

C3    661  AGCGCCTCCCTGTACGGTGCTGAACTCGAAGACATCTATCGTCAGTATTACCTGAACAGC  720
PAO1  661  AGCGCCTCCCTGTACGGCGCCGAACTCGAAGACATCTATCGCCAGTATTACCTGAACAGC  720

C3    721  AACTACACCATCCCACTGGCATCCGACCAATCGCTGGGCTTCGATTTCAACATCTACCGC  780
PAO1  721  AACTACACCATCCCACTGGCATCCGACCAATCGCTGGGCTTCGATTTCAACATCTACCGC  780

C3    781  ACAAACGATGAAGGCAAGGCCAAGGCCGGCGACATCAGCAACACCACTTGGTCCCTGGCG  840
PAO1  781  ACAAACGATGAAGGCAAGGCCAAGGCCGGCGACATCAGCAACACCACTTGGTCCCTGGCG  840

C3    841  GCAGCCTACACTCTGGATGCGCACACTTTCACCTTGGCCTACCAGAAGGTCCATAGCGAT  900
PAO1  841  GCAGCCTACACTCTGGATGCGCACACTTTCACCTTGGCCTACCAGAAGGTCCATGGCGAT  900

C3    901  CAGCCGTTTGATTATATCGGCTTCGGCGAGAACGGTTCCGGCGGCGGCGGTGACTCGATT  960
PAO1  901  CAGCCGTTTGATTATATCGGCTTCGGCCGCAACGGCTCTGGCGCAGGTGGCGACTCGATT  960

C3    961  TTCCTCGCCAACTCCGTGCAGTACTCCGACTTCAACGGCCCCGGCGAGAAATCCTGGCAG 1020
PAO1  961  TTCCTCGCCAACTCTGTCCAGTACTCCGACTTCAACGGCCCTGGCGAGAAATCCTGGCAG 1020

C3   1021  GCCCGCTACGACCTGAACCTCGCCTCCTATGGCGTTCCCGGCCTGACTTTCATGGTCCGC 1080
PAO1 1021  GCTCGCTACGACCTGAACCTAGCCTCCTATGGCGTTCCCGGCCTGACTTTCATGGTCCGC 1080

C3   1081  TATATCAATGGCAAGGACATCGATGGCACCAAGATGTCTGACAACAACGTCGGCTATAAG 1140
PAO1 1081  TATATCAATGGCAAGGACATCGATGGCACCAAGATGTCTGACAACAACGTCGGCTATAAG 1140

C3   1141  AACTACGGCTACGGCGAGGACGGCAAGCACCACGAGACCAACCTCGGAGCCAAGTACGTG 1200
PAO1 1141  AACTACGGCTACGGCGAGGATGGCAAGCACCACGAAACCAACCTCGAAGCCAAGTACGTG 1200
```

```
C3   1201 GTCCAGTCCGGTCCGGCCAAGGACCTGTCGTTCCGCATCCGCCAGGCCTGGCACCGCGCC 1260
PAO1 1201 GTCCAGTCCGGTCCGGCCAAGGACCTGTCGTTCCGCATCCGCCAGGCCTGGCACCGTGCC 1260

C3   1261 AACGCCGACCAGGCCGAAGGCGACCAGAACGAGTTCCGCCTGATCGTCGACTATCCGCTG 1320
PAO1 1261 AACGCCGACCAGGGCGAAGGCGACCAGAACGAGTTCCGCCTGATCGTCGACTATCCGCTG 1320

C3   1321 TCGATCCTGTAA  1332
PAO1 1321 TCGATCCTGTAA  1332
```

Amino acid sequence

```
C3      MKVMKWSAIALAVSAGSTQFAVADAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSG    60
PAO1    MKVMKWSAIALAVSAGSTQFAVADAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSG    60

C3      DRVDWTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKSGTGNLPVMNDGTPRDDY    120
PAO1    DRVDWTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKTGTGNLPVMNDGKPRDDY    120

C3      SRAGGAVKVRISKTMLKWGEMQPTAPVFAAGGSRLFPQTATGFQLQSSELEGLDLEAGHF    180
PAO1    SRAGGAVKVRISKTMLKWGEMQPTAPVFAAGGSRLFPQTATGFQLQSSEFEGLDLEAGHF    180

C3      TEGKQGTTTKSRGELYATYAGETAKSADFIGGRYAITDNLSASLYGAELEDIYRQYYLNS    240
PAO1    TEGKEPTTVKSRGELYATYAGETAKSADFIGGRYAITDNLSASLYGAELEDIYRQYYLNS    240

C3      NYTIPLASDQSLGFDFNIYRTNDEGKAKAGDISNTTWSLAAAYTLDAHTFTLAYQKVHSD    300
PAO1    NYTIPLASDQSLGFDFNIYRTNDEGKAKAGDISNTTWSLAAAYTLDAHTFTLAYQKVHGD    300

C3      QPFDYIGFGENGSGGGGDSIFLANSVQYSDFNGPGEKSWQARYDLNLASYGVPGLTFMVR    360
PAO1    QPFDYIGFGRNGSGAGGDSIFLANSVQYSDFNGPGEKSWQARYDLNLASYGVPGLTFMVR    360

C3      YINGKDIDGTKMSDNNVGYKNYGYGEDGKHHETNLGAKYVVQSGPAKDLSFRIRQAWHRA    420
PAO1    YINGKDIDGTKMSDNNVGYKNYGYGEDGKHHETNLEAKYVVQSGPAKDLSFRIRQAWHRA    420

C3      NADQAEGDQNEFRLIVDYPLSIL  443
PAO1    NADQGEGDQNEFRLIVDYPLSIL  443
```

C4- OprD type

Nucleotide sequence

```
C4     1 ATGAAAGTGATGAAGTGGAGCGCCATTGCACTGGCGGTTTCCGCAGGTAGCACTCAGTTC  60
PAO1   1 ATGAAAGTGATGAAGTGGAGCGCCATTGCACTGGCGGTTTCCGCAGGTAGCACTCAGTTC  60

C4    61 GCCGTGGCCGACGCATTCGTCAGCGATCAAGCCGAAGCGAAGGGGTTCATCGAAGACAGC 120
PAO1  61 GCCGTGGCCGACGCATTCGTCAGCGATCAGGCCGAAGCGAAGGGGTTCATCGAAGACAGC 120

C4   121 AGCCTCGACCTGCTGCTCCGCAACTACTATTTCAACCGTGACGGCAAGAGCGGCAGCGGG 180
PAO1 121 AGCCTCGACCTGCTGCTCCGCAACTACTATTTCAACCGTGACGGCAAGAGCGGCAGCGGG 180

C4   181 GACCGCGTCGACAGGACCCAAGGCTTCCTCACCACCTATGAATCCGGCTTCACCCAAGGC 240
PAO1 181 GACCGCGTCGACTGGACCCAAGGCTTCCTCACCACCTATGAATCCGGCTTCACCCAAGGC 240

C4   241 ACCGTCGGCTTCGGCGTCGATGCCTTCGGCTACCTCGGTCTGAAGCTCGACGGCACCTCC 300
PAO1 241 ACTGTGGGCTTCGGCGTCGATGCCTTCGGCTACCTGGGCCTGAAGCTCGACGGCACCTCC 300

C4   301 GACAAGAGCGGTACCGGCAACCTGCCAGTGATGAACGACGGCACGCCCCGTGACGACTAC 360
PAO1 301 GACAAGACCGGCACCGGCAACCTGCCGGTGATGAACGACGGCAAGCCGCGCGATGACTAC 360

C4   361 AGCCGCGCCGGTGGCGCCGTGAAGGTACGCATCTCCAAGACCATGTTGAAGTGGGGCGAG 420
PAO1 361 AGCCGCGCCGGCGGCGCCGTGAAGGTGCGCATCTCCAAGACCATGCTGAAGTGGGGCGAG 420

C4   421 ATGCAGCCGACCGCTCCGGTCTTCGCCGCCGGCGGCAGCCGCCTGTTCCCGCAGACCGCG 480
PAO1 421 ATGCAACCGACCGCCCCGGTCTTCGCCGCTGGCGGCAGCCGCCTGTTCCCGCAGACCGCG 480
```

```
C4    481 ACCGGCTTCCAATTGCAGAGCAGCGAACTCGAAGGGCTCGATCTCGAAGCGGGCCACTTC  540
PAO1  481 ACCGGCTTCCAGCTGCAGAGCAGCGAATTCGAAGGGCTCGACCTCGAGGCAGGCCACTTC  540

C4    541 ACCGAAGGCAAGCAGGGCACCACCACCAAGTCGCGCGGCGAACTCTACGCAACCTATGCA  600
PAO1  541 ACCGAGGGCAAGGAGCCGACCACCGTCAAATCGCGTGGCGAACTCTATGCCACCTACGCA  600

C4    601 GGCGAGACCGCCCAGAGCGCCGATTTCATTGGGGGCCGCTACGCAATCACCGATAACCTC  660
PAO1  601 GGCGAGACCGCCAAGAGCGCCGATTTCATTGGGGGCCGCTACGCAATCACCGATAACCTC  660

C4    661 AGCGCCTCCCTGTACGGTGCTGAACTCGAAGACATCTATCGTCAGTATTACCTGAACAGC  720
PAO1  661 AGCGCCTCCCTGTACGGCGCCGAACTCGAAGACATCTATCGCCAGTATTACCTGAACAGC  720

C4    721 AACTACACCATCCCACTGGCATCCGACCAATCGCTGGGCTTCGATTTCAACATCTACCGC  780
PAO1  721 AACTACACCATCCCACTGGCATCCGACCAATCGCTGGGCTTCGATTTCAACATCTACCGC  780

C4    781 ACAAACGATGAAGGCAAGGCCAAGGCCGGCGACATCAGCAACACCACTTGGTCCCTGGCG  840
PAO1  781 ACAAACGATGAAGGCAAGGCCAAGGCCGGCGACATCAGCAACACCACTTGGTCCCTGGCG  840

C4    841 GCAGCCTACACTCTGGATGCGCACACTTTCACCTTGGCCTACCAGAAGGTCCATGGCGAT  900
PAO1  841 GCAGCCTACACTCTGGATGCGCACACTTTCACCTTGGCCTACCAGAAGGTCCATGGCGAT  900

C4    901 CAGCCGTTTGATTATATCGGCTTCGGCGAGAACGGTTCCGGCGGCGGCGGCGACTCGATT  960
PAO1  901 CAGCCGTTTGATTATATCGGCTTCGGCCGCAACGGCTCTGGCGCAGGTGGCGACTCGATT  960

C4    961 TTCCTCGCCAACTCCGTGCAGTACTCCGACTTCAACGGCCCCGGCGAGAAATCCTGGCAG 1020
PAO1  961 TTCCTCGCCAACTCTGTCCAGTACTCCGACTTCAACGGCCCTGGCGAGAAATCCTGGCAG 1020

C4   1021 GCCCGCTACGACCTGAACCTCGCCTCCTATGGCGTTCCCGGCCTGACTTTCATGGTCCGC 1080
PAO1 1021 GCTCGCTACGACCTGAACCTAGCCTCCTATGGCGTTCCCGGCCTGACTTTCATGGTCCGC 1080

C4   1081 TATATCAATGGCAAGGACATCGATGGCACCAAGATGTCTGACAACAACGTCGGCTATAAG 1140
PAO1 1081 TATATCAATGGCAAGGACATCGATGGCACCAAGATGTCTGACAACAACGTCGGCTATAAG 1140

C4   1141 AACTACGGCTACGGCGAGGACGGCAAGCACCACGAGACCAACCTCGAAGCCAAGTACGTG 1200
PAO1 1141 AACTACGGCTACGGCGAGGATGGCAAGCACCACGAAACCAACCTCGAAGCCAAGTACGTG 1200

C4   1201 GTCCAGTCCGGTCCGGCCAAGGACCTGTCGTTCCGCATCCGCCAGGCCTGGCACCGCGCC 1260
PAO1 1201 GTCCAGTCCGGTCCGGCCAAGGACCTGTCGTTCCGCATCCGCCAGGCCTGGCACCGTGCC 1260

C4   1261 AACGCCGACCAGGCCGAAGGCGACCAGAACGAGTTCCGCCTGATCGTCGACTATCCGCTG 1320
PAO1 1261 AACGCCGACCAGGGCGAAGGCGACCAGAACGAGTTCCGCCTGATCGTCGACTATCCGCTG 1320

C4   1321 TCGATCCTGTAA  1332
PAO1 1321 TCGATCCTGTAA  1332
```

Amino acid sequence

```
C4     MKVMKWSAIALAVSAGSTQFAVADAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSG   60
PAO1   MKVMKWSAIALAVSAGSTQFAVADAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSG   60

C4     DRVDRTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKSGTGNLPVMNDGTPRDDY  120
PAO1   DRVDWTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKTGTGNLPVMNDGKPRDDY  120

C4     SRAGGAVKVRISKTMLKWGEMQPTAPVFAAGGSRLFPQTATGFQLQSSELEGLDLEAGHF  180
PAO1   SRAGGAVKVRISKTMLKWGEMQPTAPVFAAGGSRLFPQTATGFQLQSSEFEGLDLEAGHF  180


C4     TEGKQGTTTKSRGELYATYAGETAQSADFIGGRYAITDNLSASLYGAELEDIYRQYYLNS  240
PAO1   TEGKEPTTVKSRGELYATYAGETAKSADFIGGRYAITDNLSASLYGAELEDIYRQYYLNS  240

C4     NYTIPLASDQSLGFDFNIYRTNDEGKAKAGDISNTTWSLAAAYTLDAHTFTLAYQKVHGD  300
PAO1   NYTIPLASDQSLGFDFNIYRTNDEGKAKAGDISNTTWSLAAAYTLDAHTFTLAYQKVHGD  300
```

```
C4     QPFDYIGFGENGSGGGDSIFLANSVQYSDFNGPGEKSWQARYDLNLASYGVPGLTFMVR   360
PAO1   QPFDYIGFGRNGSGAGGDSIFLANSVQYSDFNGPGEKSWQARYDLNLASYGVPGLTFMVR   360

C4     YINGKDIDGTKMSDNNVGYKNYGYGEDGKHHETNLEAKYVVQSGPAKDLSFRIRQAWHRA   420
PAO1   YINGKDIDGTKMSDNNVGYKNYGYGEDGKHHETNLEAKYVVQSGPAKDLSFRIRQAWHRA   420

C4     NADQAEGDQNEFRLIVDYPLSIL  443
PAO1   NADQGEGDQNEFRLIVDYPLSIL  443
```

D1- OprD type

Nucleotide sequence

```
D1    1    ATGAAAGTGATGAAGTGGAGCGCCATTGCACTGGCGGTTTCCGCAGGTAGCACTCAGTTC   60
PAO1  1    ATGAAAGTGATGAAGTGGAGCGCCATTGCACTGGCGGTTTCCGCAGGTAGCACTCAGTTC   60

D1    61   GCCGTGGCCGACGCACTCGTCAGCGATCAGGCCGAAGCGAAGGGGTTCATCGAAGACAGC   120
PAO1  61   GCCGTGGCCGACGCATTCGTCAGCGATCAGGCCGAAGCGAAGGGGTTCATCGAAGACAGC   120

D1    121  AGCCTCGACCTGCTGCTCCGCAACTACTATTTCAACCGTGACGGCAAGAGCGGCAGCGGG   180
PAO1  121  AGCCTCGACCTGCTGCTCCGCAACTACTATTTCAACCGTGACGGCAAGAGCGGCAGCGGG   180

D1    181  GACCGCGTCGACTGGACCCAAGGCTTCCTCACCACCTATGAATCCGGCTTCACCCAAGGC   240
PAO1  181  GACCGCGTCGACTGGACCCAAGGCTTCCTCACCACCTATGAATCCGGCTTCACCCAAGGC   240

D1    241  ACCGTGGGCTTCGGCGTCGATGCCTTCGGCTACCTCGGCCTGAAGCTCGACGGCACCTCG   300
PAO1  241  ACTGTGGGCTTCGGCGTCGATGCCTTCGGCTACCTGGGCCTGAAGCTCGACGGCACCTCC   300

D1    301  GACAAGACCGGCACCGGCAACCTGCCGGTGATGAACGACGGCAAGCCGCGCGACGACTAC   360
PAO1  301  GACAAGACCGGCACCGGCAACCTGCCGGTGATGAACGACGGCAAGCCGCGCGATGACTAC   360

D1    361  AGCCGCGCTGGCGGCGCCCTGAAGGTGCGCATTTCCAAGACCATGCTGAAGTGGGGCGAA   420
PAO1  361  AGCCGCGCCGGCGGCGCCGTGAAGGTGCGCATCTCCAAGACCATGCTGAAGTGGGGCGAG   420

D1    421  ATGCAACCTACCGCGCCGGTCTTCGCCGCCGGCGGCAGCCGCCTGTTCCCGCAGACCGCG   480
PAO1  421  ATGCAACCGACCGCCCCGGTCTTCGCCGCTGGCGGCAGCCGCCTGTTCCCGCAGACCGCG   480

D1    481  ACCGGCTTCCAACTGCAGAGCAGTGAATTCGAAGGGCTCGATCTTGAAGCGGGCCACTTC   540
PAO1  481  ACCGGCTTCCAGCTGCAGAGCAGCGAATTCGAAGGGCTCGACCTCGAGGCAGGCCACTTC   540

D1    541  ACCGAAGGCAAGCAGGGCACCACCACCAAGTCGCGCGGCGAACTCTACGCAACCTATGCA   600
PAO1  541  ACCGAGGGCAAGGAGCCGACCACCGTCAAATCGCGTGGCGAACTCTATGCCACCTACGCA   600

D1    601  GGTCAGACCGCCAAGAGCGCGGACTTCGCCGGCGGCCGCTACGCGATCACCGACAATCTC   660
PAO1  601  GGCGAGACCGCCAAGAGCGCCGATTTCATTGGGGGCCGCTACGCAATCACCGATAACCTC   660

D1    661  AGCGCCTCCCTGTATGGCGCCGAATTGAAAGACATCTATCGCCAGTATTACCTGAACACC   720
PAO1  661  AGCGCCTCCCTGTACGGCGCCGAACTCGAAGACATCTATCGCCAGTATTACCTGAACAGC   720

D1    721  AACTACACCATCCCGCTGGCATCCGACCAGTCGCTGGGCTTCGACTTCAACATCTACCGC   780
PAO1  721  AACTACACCATCCCACTGGCATCCGACCAATCGCTGGGCTTCGATTTCAACATCTACCGC   780

D1    781  ACCACCGATGAAGGCAAAGCCAAGGCCGGCGACATCAGCAACACCGCCTGGTCCCTGGCC   840
PAO1  781  ACAAACGATGAAGGCAAGGCCAAGGCCGGCGACATCAGCAACACCACTTGGTCCCTGGC-   839

D1    841  GGC-GCGTACACCCTGGACGCGCACACCTTCACCCTGGCCTACCAGCAGGTGCATGGCGA   899
PAO1  840  GGCAGCCTACACTCTGGATGCGCACACTTTCACCTTGGCCTACCAGAAGGTCCATGGCGA   899

D1    900  CGAGCCGTTCGACTACATCGGTTTCGGCGAGAACCGTTCCGGCGGCGGCGGTGACTCGAT   959
PAO1  900  TCAGCCGTTTGATTATATCGGCTTCGGCCGCAACGGCTCTGGCGCAGGTGGCGACTCGAT   959

D1    960  TTTCCTCGCCAACTCCGTGCAGTACTCCGACTTCAACGGCCCCGGCGAGAAATCCTGGCA  1019
PAO1  960  TTTCCTCGCCAACTCTGTCCAGTACTCCGACTTCAACGGCCCTGGCGAGAAATCCTGGCA  1019
```

```
D1   1020 GGCCCGCTACGACCTGAACATGGCCTCCTACGGCGTTCCCGGCCTGACCTTCATGGTTCG 1079
PAO1 1020 GGCTCGCTACGACCTGAACCTAGCCTCCTATGGCGTTCCCGGCCTGACTTTCATGGTCCG 1079

D1   1080 TTACATCAACGGTAAGGACATCGACGGCACCAAG--GTC-GACTCCA-GTTCCTCCTACG 1135
PAO1 1080 CTATATCAATGGCAAGGACATCGATGGCACCAAGATGTCTGACAACAACGTCGGC-TATA 1138

D1   1136 CGGGC--CTG-TACGGCGAGGACGGCAAGCACCACGAGACCAACCTGGAAGCCAAGTACG 1192
PAO1 1139 AGAACTACGGCTACGGCGAGGATGGCAAGCACCACGAAACCAACCTCGAAGCCAAGTACG 1198

D1   1193 TCGTCCAGGCCGGCCCGGCCAAGGACCTGTCGTTCCGTATCCGCCAGGCCTGGCACCGCG 1252
PAO1 1199 TGGTCCAGTCCGGTCCGGCCAAGGACCTGTCGTTCCGCATCCGCCAGGCCTGGCACCGTG 1258

D1   1253 CCAACGCCGACGAAGGTGAAGGCGACCAGAACGAGTTCCGCCTGATCGTCGACTATCCGC 1312
PAO1 1259 CCAACGCCGACCAGGGCGAAGGCGACCAGAACGAGTTCCGCCTGATCGTCGACTATCCGC 1320

D1   1313 TGTCGATCCTGTAA  1326
PAO1 1321 TGTCGATCCTGTAA  1332
```

Amino acid sequence

```
D1    MKVMKWSAIALAVSAGSTQFAVADALVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSG   60
PAO1  MKVMKWSAIALAVSAGSTQFAVADAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSG   60

D1    DRVDWTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKTGTGNLPVMNDGKPRDDY   120
PAO1  DRVDWTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKTGTGNLPVMNDGKPRDDY   120

D1    SRAGGALKVRISKTMLKWGEMQPTAPVFAAGGSRLFPQTATGFQLQSSEFEGLDLEAGHF   180
PAO1  SRAGGAVKVRISKTMLKWGEMQPTAPVFAAGGSRLFPQTATGFQLQSSEFEGLDLEAGHF   180

D1    TEGKQGTTTKSRGELYATYAGQTAKSADFAGGRYAITDNLSASLYGAELKDIYRQYYLNT   240
PAO1  TEGKEPTTVKSRGELYATYAGETAKSADFIGGRYAITDNLSASLYGAELEDIYRQYYLNS   240


D1    NYTIPLASDQSLGFDFNIYRTTDEGKAKAGDISNTAWSLAGAYTLDAHTFTLAYQQVHGD   300
PAO1  NYTIPLASDQSLGFDFNIYRTNDEGKAKAGDISNTTWSLAAAYTLDAHTFTLAYQKVHGD   300

D1    FPFDYIGFGENRSGGGDSIFLANSVQYSDFNGPGEKSWQARYDLNMASYGVPGLTFMVR   360
PAO1  QPFDYIGFGRNGSGAGGDSIFLANSVQYSDFNGPGEKSWQARYDLNLASYGVPGLTFMVR   360

D1    YINGKDIDGTKVDSSSS-YAGL-YGEDGKHHETNLEAKYVVQAGPAKDLSFRIRQAWHRA   418
PAO1  YINGKDIDGTKMSDNNVGYKNYGYGEDGKHHETNLEAKYVVQSGPAKDLSFRIRQAWHRA   420

D1    NADEGEGDQNEFRLIVDYPLSIL  441
PAO1  NADQGEGDQNEFRLIVDYPLSIL  443
```

D2- OprD type

Nucleotide sequence

```
D2    1  ATGAAAGTGATGAAGTGGAGCGCCATTGCACTGGCGGTTTCCGCAGGTAGCACTCAGTTC   60
PAO1  1  ATGAAAGTGATGAAGTGGAGCGCCATTGCACTGGCGGTTTCCGCAGGTAGCACTCAGTTC   60

D2   61  GCCGTGGCCGACGCATTCGTCAGCGATCAGGCCGAAGCGAAGGGGTTCATCGAAGACAGC   120
PAO1 61  GCCGTGGCCGACGCATTCGTCAGCGATCAGGCCGAAGCGAAGGGGTTCATCGAAGACAGC   120

D2   121 AGCCTCGACCTGCTGCTCCGCAACTACTATTTCAACCGTGACGGCAAGGAAGGTCGGGGC   180
PAO1 121 AGCCTCGACCTGCTGCTCCGCAACTACTATTTCAACCGTGACGGCAAGAGCGGCAGCGGG   180

D2   181 GATCGCGTCGATAGGACCCAGGGCTTCCTCACCACCTACGAATCCGGCTTCACTCAGGGC   240
PAO1 181 GACCGCGTCGACTGGACCCAAGGCTTCCTCACCACCTATGAATCCGGCTTCACCCAAGGC   240
```

```
D2    241 ACCGTGGGCTTCGGCGTCGATGCCTTCGGCTACCTGGGCCTGAAGCTCGACGGTACCTCG   300
PAO1  241 ACTGTGGGCTTCGGCGTCGATGCCTTCGGCTACCTGGGCCTGAAGCTCGACGGCACCTCC   300

D2    301 GACAAGACCGGCACCGGCAACCTGCCGGTGATGAACGACGGCAAGCCGCGCGACGACTAC   360
PAO1  301 GACAAGACCGGCACCGGCAACCTGCCGGTGATGAACGACGGCAAGCCGCGCGATGACTAC   360

D2    361 AGCCGCGCTGGCGGCGCCCTGAAGGTGCGCATTTCCAAGACCATGCTGAAGTGGGGCGAA   420
PAO1  361 AGCCGCGCCGGCGGCGCCGTGAAGGTGCGCATCTCCAAGACCATGCTGAAGTGGGGCGAG   420

D2    421 ATGCAACCTACCGCGCCGGTCTTCGCCGCCGGCGGCAGCCGCCTGTTCCCGCAGACCGCG   480
PAO1  421 ATGCAACCGACCGCCCCGGTCTTCGCCGCTGGCGGCAGCCGCCTGTTCCCGCAGACCGCG   480

D2    481 ACCGGCTTCCAACTGCAGAGCAGTGAATTCGAAGGGCTCGATCTCGAAGCGGGCCACTTC   540
PAO1  481 ACCGGCTTCCAGCTGCAGAGCAGCGAATTCGAAGGGCTCGACCTCGAGGCAGGCCACTTC   540

D2    541 ACCGAAGGCAAGCAGGGCACCACCACCAAGTCGCGCGGCGAACTCTACGCAACCTATGCA   600
PAO1  541 ACCGAGGGCAAGGAGCCGACCACCGTCAAATCGCGTGGCGAACTCTATGCCACCTACGCA   600

D2    601 GGCCAGACCGCCAAGAGCGCGGACTTCGCTGGCGGCCGCTACGCGATCACCGACAATCTC   660
PAO1  601 GGCGAGACCGCCAAGAGCGCCGATTTCATTGGGGGCCGCTACGCAATCACCGATAACCTC   660


D2    661 AGCGCCTCCCTGTATGGCGCCGAATTGAAAGACATCTATCGCCAGTATTACCTGAACACC   720
PAO1  661 AGCGCCTCCCTGTACGGCGCCGAACTCGAAGACATCTATCGCCAGTATTACCTGAACAGC   720

D2    721 AACTACACAATCCCGCTGGCATCCGACCAGTCGCTGGGCTTCGACTTCAACATCTACCGC   780
PAO1  721 AACTACACCATCCCACTGGCATCCGACCAATCGCTGGGCTTCGATTTCAACATCTACCGC   780

D2    781 ACCACCGATGAAGGCAAAGCCAAGGCCGGCGACATCAGCAACACCGCCTGGTCCCTGGCC   840
PAO1  781 ACAAACGATGAAGGCAAGGCCAAGGCCGGCGACATCAGCAACACCACTTGGTCCCTGGC-   839

D2    841 GGC-GCGTACACCCTGGACGCGCACACCTTCACCCTGGCCTACCAGCAGGTGCATGGCGA   899
PAO1  840 GGCAGCCTACACTCTGGATGCGCACACTTTCACCTTGGCCTACCAGAAGGTCCATGGCGA   899

D2    900 CGAGCCGTTCGACTACATCGGCTTCGGCGAGAACGGTTCGGGCGGCGGCGGTGACTCGAT   959
PAO1  900 TCAGCCGTTTGATTATATCGGCTTCGGCCGCAACGGCTCTGGCGCAGGTGGCGACTCGAT   959

D2    960 TTTCCTCGCCAACTCCGTGCAGTACTCCGACTTCAACGGCCCGGGCGAGAAATCCTGGCA  1019
PAO1  960 TTTCCTCGCCAACTCTGTCCAGTACTCCGACTTCAACGGCCCTGGCGAGAAATCCTGGCA  1019

D2   1020GGCCCGCTACGACCTGAACATGGCCTCCTACGGCGTTCCCGGCCTGACCTTCATGGTTCG  1079
PAO1 1020GGCTCGCTACGACCTGAACCTAGCCTCCTATGGCGTTCCCGGCCTGACTTTCATGGTCCG  1079

D2   1080TTACATCAACGGTAAGGACATCGACGGCACCAAG--GTC-GACTCCA-GTTCATCCTACG  1135
PAO1 1080CTATATCAATGGCAAGGACATCGATGGCACCAAGATGTCTGACAACAACGTCGGC-TATA  1138

D2   1136CGGGC--CTG-TACGGCGAGGACGGCAAGCACCACGAGACCAACCTGGAAGCCAAATACG  1192
PAO1 1139AGAACTACGGCTACGGCGAGGATGGCAAGCACCACGAAACCAACCTCGAAGCCAAGTACG  1198

D2   1193TCGTCCAGGCCGGCCCGGCCAAGGACCTGTCGTTCCGTATCCGCCAGGCCAGGCACCGCG  1252
PAO1 1199TGGTCCAGTCCGGTCCGGCCAAGGACCTGTCGTTCCGCATCCGCCAGGCCTGGCACCGTG  1258

D2   1253CCAACGCCGACGAGGGTGAAGGCGACCAGAACGAGTTCCGCCTGATCGTCGACTATCCGC  1312
PAO1 1259CCAACGCCGACCAGGGCGAAGGCGACCAGAACGAGTTCCGCCTGATCGTCGACTATCCGC  1320

D2   1313TGTCGATCCTGTAA  1326
PAO1 1321TGTCGATCCTGTAA  1332
```

Amino acid sequence

```
D2     MKVMKWSAIALAVSAGSTQFAVADAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKEGRG    60
PAO1   MKVMKWSAIALAVSAGSTQFAVADAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSG    60

D2     DRVDRTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKTGTGNLPVMNDGKPRDDY    120
PAO1   DRVDWTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKTGTGNLPVMNDGKPRDDY    120

D2     SRAGGALKVRISKTMLKWGEMQPTAPVFAAGGSRLFPQTATGFQLQSSEFEGLDLEAGHF    180
PAO1   SRAGGAVKVRISKTMLKWGEMQPTAPVFAAGGSRLFPQTATGFQLQSSEFEGLDLEAGHF    180

D2      TEGKQGTTTKSRGELYATYAGQTAKSADFAGGRYAITDNLSASLYGAELKDIYRQYYLNT    240
PAO1    TEGKEPTTVKSRGELYATYAGETAKSADFIGGRYAITDNLSASLYGAELEDIYRQYYLNS    240

D2      NYTIPLASDQSLGFDFNIYRTTDEGKAKAGDISNTAWSLAGAYTLDAHTFTLAYQQVHGD    300
PAO1    NYTIPLASDQSLGFDFNIYRTNDEGKAKAGDISNTTWSLAAAYTLDAHTFTLAYQKVHGD    300

D2      EPFDYIGFGENGSGGGGDSIFLANSVQYSDFNGPGEKSWQARYDLNMASYGVPGLTFMVR    360
PAO1    QPFDYIGFGRNGSGAGGDSIFLANSVQYSDFNGPGEKSWQARYDLNLASYGVPGLTFMVR    360

D2      YINGKDIDGTKVDSSSS-YAGL-YGEDGKHHETNLEAKYVVQAGPAKDLSFRIRQARHRA    418
PAO1    YINGKDIDGTKMSDNNVGYKNYGYGEDGKHHETNLEAKYVVQSGPAKDLSFRIRQAWHRA    420

D2      NADEGEGDQNEFRLIVDYPLSIL  441
PAO1    NADQGEGDQNEFRLIVDYPLSIL  443
```

D3- OprD type

Nucleotide sequence

```
D3     1   ATGAAAGTGATGAAGTGGAGCGCCATTGCACTGGCGGTTTCCGCAGGTAGCACTCAGTTC   60
PAO1   1   ATGAAAGTGATGAAGTGGAGCGCCATTGCACTGGCGGTTTCCGCAGGTAGCACTCAGTTC   60

D3    61   GCCGTGGCCGACGCATTCGTCAGCGATCAGGCCGAAGCGAAGGGGTTCATCGAAGACAGC  120
PAO1  61   GCCGTGGCCGACGCATTCGTCAGCGATCAGGCCGAAGCGAAGGGGTTCATCGAAGACAGC  120

D3   121   AGCCTCGACCTGCTGCTCCGCAACTACTATTTCAACCGTGACGGCAAGGAAGGTCGGGGC  180
PAO1 121   AGCCTCGACCTGCTGCTCCGCAACTACTATTTCAACCGTGACGGCAAGAGCGGCAGCGGG  180


D3   181   GATCGCGTCGATTGGACCCAGGGCTTCCTCACCACCTACGAATCCGGCTTCACTCAAGGC  240
PAO1 181   GACCGCGTCGACTGGACCCAAGGCTTCCTCACCACCTATGAATCCGGCTTCACCCAAGGC  240

D3   241   ACCGTGGGCTTCGGCGTCGATGCCTTCGGCTACCTGGGCCTGAAACTCGACGGTACCTCG  300
PAO1 241   ACTGTGGGCTTCGGCGTCGATGCCTTCGGCTACCTGGGCCTGAAGCTCGACGGCACCTCC  300

D3   301   GACAAGACCGGCACCGGCAACCTGCCGGTGATGAACGACGGCAAGCCGCGCGACGACTAC  360
PAO1 301   GACAAGACCGGCACCGGCAACCTGCCGGTGATGAACGACGGCAAGCCGCGCGATGACTAC  360

D3   361   AGCCGCGCTGGCGGCGCCCTGAAGGTGCGCATTTCCAAGACCATGCTGAAGTGGGGCGAA  420
PAO1 361   AGCCGCGCCGGCGGCGCCGTGAAGGTGCGCATCTCCAAGACCATGCTGAAGTGGGGCGAG  420

D3   421   ATGCAACCTACCGCGCCGGTCTTCGCCGCCGGCGGCAGCCGCCTGTTCCCGCAGACCGCG  480
PAO1 421   ATGCAACCGACCGCCCCGGTCTTCGCCGCTGGCGGCAGCCGCCTGTTCCCGCAGACCGCG  480

D3   481   ACCGGCTTCCAACTGCAGAGCAGTGAATTCGAAGGGCTCGATCTCGAAGCGGGCCACTTC  540
PAO1 481   ACCGGCTTCCAGCTGCAGAGCAGCGAATTCGAAGGGCTCGACCTCGAGGCAGGCCACTTC  540

D3   541   ACCGAAGGCAAGCAGGGCACCACCACCAAGTCGCCGGCGAACTCTACGCAACCTATGCA  600
PAO1 541   ACCGAGGGCAAGGAGCCGACCACCGTCAAATCGCGTGGCGAACTCTATGCCACCTACGCA  600
```

```
D3    601  GGCCAGACCGCCAAGAGCGCGGACTTCGCCGGCGGCCGCTACGCGATCACCGACAATCTC  660
PAO1  601  GGCGAGACCGCCAAGAGCGCCGATTTCATTGGGGGCCGCTACGCAATCACCGATAACCTC  660

D3    661  AGCGCCTCCCTGTATGGCGCCGAATTGAAAGACATCTATCGCCAGTATTACCTGAACACC  720
PAO1  661  AGCGCCTCCCTGTACGGCGCCGAACTCGAAGACATCTATCGCCAGTATTACCTGAACAGC  720

D3    721  AACTACACAATCCCGCTGGCATCCGACCAGTCGCTGGGCTTCGACTTCAACATCTACCGC  780
PAO1  721  AACTACACCATCCCACTGGCATCCGACCAATCGCTGGGCTTCGATTTCAACATCTACCGC  780

D3    781  ACCACCGATGAAGGCAAAGCCAAGGCCGGCGACATCAGCAACACCGCCTGGTCCCTGGCC  840
PAO1  781  ACAAACGATGAAGGCAAGGCCAAGGCCGGCGACATCAGCAACACCACTTGGTCCCTGGC-  839

D3    841  GGC-GCGTACACCCTGGACGCGCACACCTTCACCCTGGCCTACCAGCAGGTGCATGGCGA  899
PAO1  840  GGCAGCCTACACTCTGGATGCGCACACTTTCACCTTGGCCTACCAGAAGGTCCATGGCGA  899

D3    900  CGAGCCGTTCGACTACATCGGCTTCGGCGAGAACGGTTCGGGCGGCGGCGGTGACTCGAT  959
PAO1  900  TCAGCCGTTTGATTATATCGGCTTCGGCCGCAACGGCTCTGGCGCAGGTGGCGACTCGAT  959

D3    960  TTTCCTCGCCAACTCCGTGCAGTACTCCGACTTCAACGGCCCGGGCGAGAAATCCTGGCA 1019
PAO1  960  TTTCCTCGCCAACTCTGTCCAGTACTCCGACTTCAACGGCCCTGGCGAGAAATCCTGGCA 1019

D3   1020  GGCCCGCTACGACCTGAACATGGCCTCCTACGGCGTTCCCGGCCTGACCTTCATGGTTCG 1079
PAO1 1020  GGCTCGCTACGACCTGAACCTAGCCTCCTATGGCGTTCCCGGCCTGACTTTCATGGTCCG 1079

D3   1080  TTACATCAACGGTAAGGACATCGACGGCACCAAG--GTC-GACTCCA-GTTCCTCCTACG 1135
PAO1 1080  CTATATCAATGGCAAGGACATCGATGGCACCAAGATGTCTGACAACAACGTCGGC-TATA 1138

D3   1136  CGGGC--CTG-TACGGCGAGGACGGCAAGCACCACGAGACCAACCTGGAAGCCAAATACG 1192
PAO1 1139  AGAACTACGGCTACGGCGAGGATGGCAAGCACCACGAAACCAACCTCGAAGCCAAGTACG 1198

D3   1193  TCGTCCAGGCCGGCCCGGCCAAGGACCTGTCGTTCCGTATCCGCCAGGCCTGGCACCGCG 1252
PAO1 1199  TGGTCCAGTCCGGTCCGGCCAAGGACCTGTCGTTCCGCATCCGCCAGGCCTGGCACCGTG 1258

D3   1253  CCAATGCCGACGAGGGTGAAGGCGACCAGAACGAGTTCCGCCTGATCGTCGACTATCCGC 1312
PAO1 1259  CCAACGCCGACCAGGGCGAAGGCGACCAGAACGAGTTCCGCCTGATCGTCGACTATCCGC 1320

D3   1313  TGTCGATCCTGTAA  1326
PAO1 1321  TGTCGATCCTGTAA  1332
```

Amino acid sequence

```
D3     MKVMKWSAIALAVSAGSTQFAVADAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKEGRG    60
PAO1   MKVMKWSAIALAVSAGSTQFAVADAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSG    60

D3     DRVDWTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKTGTGNLPVMNDGKPRDDY    120
PAO1   DRVDWTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKTGTGNLPVMNDGKPRDDY    120

D3     SRAGGALKVRISKTMLKWGEMQPTAPVFAAGGSRLFPQTATGFQLQSSEFEGLDLEAGHF    180
PAO1   SRAGGAVKVRISKTMLKWGEMQPTAPVFAAGGSRLFPQTATGFQLQSSEFEGLDLEAGHF    180

D3      TEGKQGTTTKSRGELYATYAGQTAKSADFAGGRYAITDNLSASLYGAELKDIYRQYYLNT    240
PAO1    TEGKEPTTVKSRGELYATYAGETAKSADFIGGRYAITDNLSASLYGAELEDIYRQYYLNS    240

D3      NYTIPLASDQSLGFDFNIYRTTDEGKAKAGDISNTAWSLAGAYTLDAHTFTLAYQQVHGD    300
PAO1    NYTIPLASDQSLGFDFNIYRTNDEGKAKAGDISNTTWSLAAAYTLDAHTFTLAYQKVHGD    300

D3      FPFDYIGFGENGSGGGGDSIFLANSVQYSDFNGPGEKSWQARYDLNMASYGVPGLTFMVR    360
PAO1    QPFDYIGFGRNGSGAGGDSIFLANSVQYSDFNGPGEKSWQARYDLNLASYGVPGLTFMVR    360

D3      YINGKDIDGTKVDSSSS-YAGL-YGEDGKHHETNLEAKYVVQAGPAKDLSFRIRQAWHRA    418
PAO1    YINGKDIDGTKMSDNNVGYKNYGYGEDGKHHETNLEAKYVVQSGPAKDLSFRIRQAWHRA    420

D3      NADEGEGDQNEFRLIVDYPLSIL  441
PAO1    NADQGEGDQNEFRLIVDYPLSIL  443
```

D4- OprD type

Nucleotide sequence

```
D4     1   ATGAAAGTGATGAAGTGGAGCGCCATTGCACTGGCGGTTTCCGCAGGTAGCACTCAGTTC  60
PAO1   1   ATGAAAGTGATGAAGTGGAGCGCCATTGCACTGGCGGTTTCCGCAGGTAGCACTCAGTTC  60

D4    61   GCCGTGGCCGACGCATTCGTCAGCGATCAGGCCGAAGCGAAGGGGTTCATCGAAGACAGC  120
PAO1  61   GCCGTGGCCGACGCATTCGTCAGCGATCAGGCCGAAGCGAAGGGGTTCATCGAAGACAGC  120

D4   121   AGCCTCGACCTGCTGCTCCGCAACTACTATTTCAACCGTGACGGCAAGAGCGGCAGCGGG  180
PAO1 121   AGCCTCGACCTGCTGCTCCGCAACTACTATTTCAACCGTGACGGCAAGAGCGGCAGCGGG  180

D4   181   GACCGCGTCGACTGGACCCAAGGCTTCCTCACCACCTATGAATCCGGCTTCACCCAAGGC  240
PAO1 181   GACCGCGTCGACTGGACCCAAGGCTTCCTCACCACCTATGAATCCGGCTTCACCCAAGGC  240

D4   241   ACCGTGGGCTTCGGCGTCGATGCCTTCGGCTACCTCGGCCTGAAGCTCGACGGCACCTCG  300
PAO1 241   ACTGTGGGCTTCGGCGTCGATGCCTTCGGCTACCTGGGCCTGAAGCTCGACGGCACCTCC  300

D4   301   GACAAGACCGGCACCGGCAACCTGCCGGTGATGAACGACGGCAAGCCGCGCGACGACTAC  360
PAO1 301   GACAAGACCGGCACCGGCAACCTGCCGGTGATGAACGACGGCAAGCCGCGCGATGACTAC  360

D4   361   AGCCGCGCTGGCGGCGCCCTGAAGGTGCGCATTTCCAAGACCATGCTGAAGTGGGGCGAA  420
PAO1 361   AGCCGCGCCGGCGGCGCCGTGAAGGTGCGCATCTCCAAGACCATGCTGAAGTGGGGCGAG  420

D4   421   ATGCAACCTACCGCGCCGGTCTTCGCCGCCGGCGGCAGCCGCCTGTTCCCGCAGACCGCG  480
PAO1 421   ATGCAACCGACCGCCCCGGTCTTCGCCGCTGGCGGCAGCCGCCTGTTCCCGCAGACCGCG  480

D4   481   ACCGGCTTCCAACTGCAGAGCAGTGAATTCGAAGGGCTCGATCTCGAAGCGGGCCACTTC  540
PAO1 481   ACCGGCTTCCAGCTGCAGAGCAGCGAATTCGAAGGGCTCGACCTCGAGGCAGGCCACTTC  540

D4   541   ACCGAAGGCAAGCAGGGCACCACCACCAAGTCGCGCGGCGAACTCTACGCAACCTATGCA  600
PAO1 541   ACCGAGGGCAAGGAGCCGACCACCGTCAAATCGCGTGGCGAACTCTATGCCACCTACGCA  600

D4   601   GGTCAGACCGCCAAGAGCGCGGACTTCGCCGGCGGCCGCTACGCGATCACCGACAATCTC  660
PAO1 601   GGCGAGACCGCCAAGAGCGCCGATTTCATTGGGGGCCGCTACGCAATCACCGATAACCTC  660

D4   661   AGCGCCTCCCTGTATGGCGCCGAATTGAAAGACATCTATCGCCAGTATTACCTGAACACC  720
PAO1 661   AGCGCCTCCCTGTACGGCGCCGAACTCGAAGACATCTATCGCCAGTATTACCTGAACAGC  720

D4   721   AACTACACCATCCCGCTGGCATCCGACCAGTCGCTGGGCTTCGACTTCAACATCTACCGC  780
PAO1 721   AACTACACCATCCCACTGGCATCCGACCAATCGCTGGGCTTCGATTTCAACATCTACCGC  780

D4   781   ACCACCGATGAAGGCAAAGCCAAGGCCGGCGACATCAGCAACACCGCCTGGTCCCTGGCC  840
PAO1 781   ACAAACGATGAAGGCAAGGCCAAGGCCGGCGACATCAGCAACACCACTTGGTCCCTGGC-  839

D4   841   GGC-GCGTACACCCTGGACGCGCACACCTTCACCCTGGCCTACCAGCAGGTGCATGGCGA  899
PAO1 840   GGCAGCCTACACTCTGGATGCGCACACTTTCACCTTGGCCTACCAGAAGGTCCATGGCGA  899

D4   900   CGAGCCGTTCGACTACATCGGCTTCGGCGAGAACCGTTCCGGCGGCGGCGGTGACTCGAT  959
PAO1 900   TCAGCCGTTTGATTATATCGGCTTCGGCCGCAACGGCTCTGGCGCAGGTGGCGACTCGAT  959

D4   960   TTTCCTCGCCAACTCCGTGCAGTACTCCGACTTCAACGGCCCCGGCGAGAAATCCCGGCA 1019
PAO1 960   TTTCCTCGCCAACTCTGTCCAGTACTCCGACTTCAACGGCCCTGGCGAGAAATCCTGGCA 1019

D4   1020  GGCCCGCTACGACCTGAACATGGCCTCCTACGGCGTTCCCGGCCTGACCTTCATGGTTCG 1079
PAO1 1020  GGCTCGCTACGACCTGAACCTAGCCTCCTATGGCGTTCCCGGCCTGACTTTCATGGTCCG 1079

D4   1080  TTACATCAACGGTAAGGACATCGACGGCACCAAG--GTC-GACTCCA-GTTCCTCCTACG 1135
PAO1 1080  CTATATCAATGGCAAGGACATCGATGGCACCAAGATGTCTGACAACAACGTCGGC-TATA 1138
```

```
D4   1136 CGGGC--CTG-TACGGCGAGGACGGCAAGCACCACGAGACCAACCTGGAAGCCAAGTACG 1192
PAO1 1139 AGAACTACGGCTACGGCGAGGATGGCAAGCACCACGAAACCAACCTCGAAGCCAAGTACG 1198

D4   1193 TCGTCCAGGCCGGCCCGGCCAAGGACCTGTCGTTCCGTATCCGCCAGGCCTGGCACCGCG 1252
PAO1 1199 TGGTCCAGTCCGGTCCGGCCAAGGACCTGTCGTTCCGCATCCGCCAGGCCTGGCACCGTG 1258

D4   1253 CCAACGCCGACGAAGGTGAAGGCGACCAGAACGAGTTCCGCCTGATCGTCGACTATCCGC 1312
PAO1 1259 CCAACGCCGACCAGGGCGAAGGCGACCAGAACGAGTTCCGCCTGATCGTCGACTATCCGC 1320

D4   1313 TGTCGATCCTGTAA  1326
PAO1 1321 TGTCGATCCTGTAA  1332
```

Amino acid sequence

```
D4     MKVMKWSAIALAVSAGSTQFAVADAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSG   60
PAO1   MKVMKWSAIALAVSAGSTQFAVADAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSG   60

D4     DRVDWTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKTGTGNLPVMNDGKPRDDY   120
PAO1   DRVDWTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKTGTGNLPVMNDGKPRDDY   120

D4     SRAGGALKVRISKTMLKWGEMQPTAPVFAAGGSRLFPQTATGFQLQSSEFEGLDLEAGHF   180
PAO1   SRAGGAVKVRISKTMLKWGEMQPTAPVFAAGGSRLFPQTATGFQLQSSEFEGLDLEAGHF   180

D4     TEGKQGTTTKSRGELYATYAGQTAKSADFAGGRYAITDNLSASLYGAELKDIYRQYYLNT   240
PAO1   TEGKEPTTVKSRGELYATYAGETAKSADFIGGRYAITDNLSASLYGAELEDIYRQYYLNS   240

D4     NYTIPLASDQSLGFDFNIYRTTDEGKAKAGDISNTAWSLAGAYTLDAHTFTLAYQQVHGD   300
PAO1   NYTIPLASDQSLGFDFNIYRTNDEGKAKAGDISNTTWSLAAAYTLDAHTFTLAYQKVHGD   300

D4     EPFDYIGFGENRSGGGGDSIFLANSVQYSDFNGPGEKSRQARYDLNMASYGVPGLTFMVR   360
PAO1   QPFDYIGFGRNGSGAGGDSIFLANSVQYSDFNGPGEKSWQARYDLNLASYGVPGLTFMVR   360

D4     YINGKDIDGTKVDSSSS-YAGL-YGEDGKHHETNLEAKYVVQAGPAKDLSFRIRQAWHRA   418
PAO1   YINGKDIDGTKMSDNNVGYKNYGYGEDGKHHETNLEAKYVVQSGPAKDLSFRIRQAWHRA   420

D4     NADEGEGDQNEFRLIVDYPLSIL  441
PAO1   NADQGEGDQNEFRLIVDYPLSIL  443
```

D5- OprD type

Nucleotide sequence

```
D5     1   ATGAAAGTGATGAAGTGGAGCGCCATTGCACTGGCGGTTTCCGCAGGTAGCACTCAGTTC  60
PAO1   1   ATGAAAGTGATGAAGTGGAGCGCCATTGCACTGGCGGTTTCCGCAGGTAGCACTCAGTTC  60

D5     61  GCCGTGGCCGACGCATTCGTCAGCGATCAGGCCGAAGCGAAGGGGTTCATCGAAGACAGC  120
PAO1   61  GCCGTGGCCGACGCATTCGTCAGCGATCAGGCCGAAGCGAAGGGGTTCATCGAAGACAGC  120


D5     121 AGCCTCGACCTGCTGCTCCGCAACTACTATTTCAACCGTGACGGCAAGAGCGGCAGCGGG  180
PAO1   121 AGCCTCGACCTGCTGCTCCGCAACTACTATTTCAACCGTGACGGCAAGAGCGGCAGCGGG  180

D5     181 GACCGCGTCGACTGGACCCAAGGCTTCCTCACCACCTATGAATCCGGCTTCACCCAAGGC  240
PAO1   181 GACCGCGTCGACTGGACCCAAGGCTTCCTCACCACCTATGAATCCGGCTTCACCCAAGGC  240

D5     241 ACCGTGGGCTTCGGCGTCGATGCCTTCGGCTACCTCGGCCTGAAGCTCGACGGCACCTCG  300
PAO1   241 ACTGTGGGCTTCGGCGTCGATGCCTTCGGCTACCTGGGCCTGAAGCTCGACGGCACCTCC  300

D5     301 GACAAGACCGGCACCGGCAACCTGCCGGTGATGAACGACGGCAAGCCGCGCGACGACTAC  360
PAO1   301 GACAAGACCGGCACCGGCAACCTGCCGGTGATGAACGACGGCAAGCCGCGCGATGACTAC  360

D5     361 AGCCGCGCTGGCGGCGCCCTGAAGGTGCGCATTTCCAAGACCATGCTGAAGTGGGGCGAA  420
PAO1   361 AGCCGCGCCGGCGGCGCCGTGAAGGTGCGCATCTCCAAGACCATGCTGAAGTGGGGCGAG  420
```

```
D5    421  ATGCAACCTACCGCGCCGGTCTTCGCCGCCGGCGGCAGCCGCCTGTTCCCGCAGACCGCG  480
PAO1  421  ATGCAACCGACCGCCCCGGTCTTCGCCGCTGGCGGCAGCCGCCTGTTCCCGCAGACCGCG  480

D5    481  ACCGGCTTCCAACTGCAGAGCAGTGAATTCGAAGGGCTCGATCTTGAAGCGGGCCACTTC  540
PAO1  481  ACCGGCTTCCAGCTGCAGAGCAGCGAATTCGAAGGGCTCGACCTCGAGGCAGGCCACTTC  540

D5    541  ACCGAAGGCAAGCAGGGCACCACCACCAAGTCGCGCGGCGAACTCTACGCAACCTATGCA  600
PAO1  541  ACCGAGGGCAAGGAGCCGACCACCGTCAAATCGCGTGGCGAACTCTATGCCACCTACGCA  600

D5    601  GGTCAGACCGCCAAGAGCGCGGACTTCGCCGGCGGCCGCTACGCGATCACCGACAATCTC  660
PAO1  601  GGCGAGACCGCCAAGAGCGCCGATTTCATTGGGGGCCGCTACGCAATCACCGATAACCTC  660

D5    661  AGCGCCTCCCTGTATGGCGCCGAATTGAAAGACATCTATCGCCAGTATTACCTGAACACC  720
PAO1  661  AGCGCCTCCCTGTACGGCGCCGAACTCGAAGACATCTATCGCCAGTATTACCTGAACAGC  720

D5    721  AACTACACCATCCCGCTGGCATCCGACCAGTCGCTGGGCTTCGACTTCAACATCTACCGC  780
PAO1  721  AACTACACCATCCCACTGGCATCCGACCAATCGCTGGGCTTCGATTTCAACATCTACCGC  780

D5    781  ACCACCGATGAAGGCAAAGCCAAGGCCGGCGACATCAGCAACACCGCCTGGTCCCTGGCC  840
PAO1  781  ACAAACGATGAAGGCAAGGCCAAGGCCGGCGACATCAGCAACACCACTTGGTCCCTGGC-  839

D5    841  GGC-GCGTACACCCTGGACGCGCACACCTTCACCCTGGCCTACCAGCAGGTGCATGGCGA  899
PAO1  840  GGCAGCCTACACTCTGGATGCGCACACTTTCACCTTGGCCTACCAGAAGGTCCATGGCGA  899

D5    900  CGAGCCGTTCGACTACATCGGCTTCGGCGAGAACCGTTCCGGCGGCGGCGGTGACTCGAT  959
PAO1  900  TCAGCCGTTTGATTATATCGGCTTCGGCCGCAACGGCTCTGGCGCAGGTGGCGACTCGAT  959

D5    960  TTTCCTCGCCAACTCCGTGCAGTACTCCGACTTCAACGGCCCCGGCGAGAAATCCTGGCA 1019
PAO1  960  TTTCCTCGCCAACTCTGTCCAGTACTCCGACTTCAACGGCCCTGGCGAGAAATCCTGGCA 1019

D5   1020  GGCCCGCTACGACCTGAACATGGCCTCCTACGGCGTTCCCGGCCTGACCTTCATGGTTCG 1079
PAO1 1020  GGCTCGCTACGACCTGAACCTAGCCTCCTATGGCGTTCCCGGCCTGACTTTCATGGTCCG 1079

D5   1080  TTACATCAACGGTAAGGACATCGACGGCACCAAG--GTC-GACTCCA-GTTCCTCCTACG 1135
PAO1 1080  CTATATCAATGGCAAGGACATCGATGGCACCAAGATGTCTGACAACAACGTCGGC-TATA 1138

D5   1136  CGGGC--CTG-TACGGCGAGGACGGCAAGCACCACGAGACCAACCTGGAAGCCAAGTACG 1192
PAO1 1139  AGAACTACGGCTACGGCGAGGATGGCAAGCACCACGAAACCAACCTCGAAGCCAAGTACG 1198

D5   1193  TCGTCCAGGCCGGCCCGGCCAAGGACCTGTCGTTCCGTATCCGCCAGGCCTGGCACCGCG 1252
PAO1 1199  TGGTCCAGTCCGGTCCGGCCAAGGACCTGTCGTTCCGCATCCGCCAGGCCTGGCACCGTG 1258

D5   1253  CCAATGCCGACGAAGGTGAAGGCGACCAGAACGAGTTCCGCCTGATCGTCGACTATCCGC 1312
PAO1 1259  CCAACGCCGACCAGGGCGAAGGCGACCAGAACGAGTTCCGCCTGATCGTCGACTATCCGC 1320

D5   1313  TGTCGATCCTGTAA  1326
PAO1 1321  TGTCGATCCTGTAA  1332
```

Amino acid sequence

```
D5     MKVMKWSAIALAVSAGSTQFAVADAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSG   60
PAO1   MKVMKWSAIALAVSAGSTQFAVADAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSG   60

D5     DRVDWTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKTGTGNLPVMNDGKPRDDY  120
PAO1   DRVDWTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKTGTGNLPVMNDGKPRDDY  120

D5     SRAGGALKVRISKTMLKWGEMQPTAPVFAAGGSRLFPQTATGFQLQSSEFEGLDLEAGHF  180
PAO1   SRAGGAVKVRISKTMLKWGEMQPTAPVFAAGGSRLFPQTATGFQLQSSEFEGLDLEAGHF  180

D5     TEGKQGTTTKSRGELYATYAGQTAKSADFAGGRYAITDNLSASLYGAELKDIYRQYYLNT  240
PAO1   TEGKEPTTVKSRGELYATYAGETAKSADFIGGRYAITDNLSASLYGAELEDIYRQYYLNS  240

D5     NYTIPLASDQSLGFDFNIYRTTDEGKAKAGDISNTAWSLAGAYTLDAHTFTLAYQQVHGD  300
PAO1   NYTIPLASDQSLGFDFNIYRTNDEGKAKAGDISNTTWSLAAAYTLDAHTFTLAYQKVHGD  300
```

```
D5     EPFDYIGFGENRSGGGDSIFLANSVQYSDFNGPGEKSWQARYDLNMASYGVPGLTFMVR   360
PAO1   QPFDYIGFGRNGSGAGGDSIFLANSVQYSDFNGPGEKSWQARYDLNLASYGVPGLTFMVR   360

D5     YINGKDIDGTKVDSSSS-YAGL-YGEDGKHHETNLEAKYVVQAGPAKDLSFRIRQAWHRA   418
PAO1   YINGKDIDGTKMSDNNVGYKNYGYGEDGKHHETNLEAKYVVQSGPAKDLSFRIRQAWHRA   420

D5     NADEGEGDQNEFRLIVDYPLSIL  441
PAO1   NADQGEGDQNEFRLIVDYPLSIL  443
```

D6- OprD type

Nucleotide sequence

```
D6     1  ATGAAAGTGATGAAGTGGAGCGCCATTGCACTGGCGGTTTCCGCAGGTAGCACTCAGTTC   60
PAO1   1  ATGAAAGTGATGAAGTGGAGCGCCATTGCACTGGCGGTTTCCGCAGGTAGCACTCAGTTC   60

D6    61 GCCGTGGCCGACGCATTCGTCAGCGATCAGGCCGAAGCGAAGGGGTTCATCGAAGACAGC   120
PAO1  61 GCCGTGGCCGACGCATTCGTCAGCGATCAGGCCGAAGCGAAGGGGTTCATCGAAGACAGC   120

D6   121 AGCCTCGACCTGCTGCTCCGCAACTACTATTTCAACCGTGACGGCAAGAGCGGCAGCGGG   180
PAO1 121 AGCCTCGACCTGCTGCTCCGCAACTACTATTTCAACCGTGACGGCAAGAGCGGCAGCGGG   180

D6   181 GACCGCGTCGACTGGACCCAAGGCTTCCTCACCACCTATGAATCCGGCTTCACCCAAGGC   240
PAO1 181 GACCGCGTCGACTGGACCCAAGGCTTCCTCACCACCTATGAATCCGGCTTCACCCAAGGC   240

D6   241 ACCGTGGGCTTCGGCGTCGATGCCTTCGGCTACCTCGGCCTGAAGCTCGACGGCACCTCG   300
PAO1 241 ACTGTGGGCTTCGGCGTCGATGCCTTCGGCTACCTGGGCCTGAAGCTCGACGGCACCTCC   300

D6   301 GACAAGACCGGCACCGGCAACCTGCCGGTGATGAACGACGGCAAGCCGCGCGACGACTAC   360
PAO1 301 GACAAGACCGGCACCGGCAACCTGCCGGTGATGAACGACGGCAAGCCGCGCGATGACTAC   360

D6   361 AGCCGCGCTGGCGGCGCCCTGAAGGTGCGCATTTCCAAGACCATGCTGAAGTGGGGCGAA   420
PAO1 361 AGCCGCGCCGGCGGCGCCGTGAAGGTGCGCATCTCCAAGACCATGCTGAAGTGGGGCGAG   420

D6   421 ATGCAACCTACCGCGCCGGTCTTCGCCGCCGGCGGCAGCCGCCTGTTCCCGCAGACCGCG   480
PAO1 421 ATGCAACCGACCGCCCCGGTCTTCGCCGCTGGCGGCAGCCGCCTGTTCCCGCAGACCGCG   480

D6   481 ACCGGCTTCCAACTGCAGAGCAGTGAATTCGAAGGGCTCGATCTCGAAGCGGGCCACTTC   540
PAO1 481 ACCGGCTTCCAGCTGCAGAGCAGCGAATTCGAAGGGCTCGACCTCGAGGCAGGCCACTTC   540

D6   541 ACCGAAGGCAAGCAGGGCACCACCACCAAGTCGCGCGGCGAACTCTACGCAACCTATGCA   600
PAO1 541 ACCGAGGGCAAGGAGCCGACCACCGTCAAATCGCGTGGCGAACTCTATGCCACCTACGCA   600

D6   601 GGTCAGACCGCCAAGAGCGCGGACTTCGCCGGCGGCCGCTACGCGATCACCGACAATCTC   660
PAO1 601 GGCGAGACCGCCAAGAGCGCCGATTTCATTGGGGGCCGCTACGCAATCACCGATAACCTC   660


D6   661 AGCGCCTCCCTGTATGGCGCCGAATTGAAAGACATCTATCGCCAGTATTACCTGAACACC   720
PAO1 661 AGCGCCTCCCTGTACGGCGCCGAACTCGAAGACATCTATCGCCAGTATTACCTGAACAGC   720

D6   721 AACTACACCATCCCGCTGGCATCCGACCAGTCGCTGGGCTTCGACTTCAACATCTACCGC   780
PAO1 721 AACTACACCATCCCACTGGCATCCGACCAATCGCTGGGCTTCGATTTCAACATCTACCGC   780

D6   781 ACCACCGATGAAGGCAAAGCCAAGGCCGGCGACATCAGCAACACCGCCTGGTCCCTGGCC   840
PAO1 781 ACAAACGATGAAGGCAAGGCCAAGGCCGGCGACATCAGCAACACCACTTGGTCCCTGGC-   839

D6   841 GGC-GCGTACACCCTGGACGCGCACACCTTCACCCTGGCCTACCAGCAGGTGCATGGCGA   899
PAO1 840 GGCAGCCTACACTCTGGATGCGCACACTTTCACCTTGGCCTACCAGAAGGTCCATGGCGA   899

D6   900  CGAGCCGTTCGACTACATCGGCTTCGGCCGCAACCGTTCCGGCGGCGGCGGTGACTCGAT   959
PAO1 900  TCAGCCGTTTGATTATATCGGCTTCGGCCGCAACGGCTCTGGCGCAGGTGGCGACTCGAT   959

D6   960  TTTCCTCGCCAACTCCGTGCAGTACTCCGACTTCAACGGCCCGGGCGAGAAATCCTGGCA  1019
PAO1 960  TTTCCTCGCCAACTCTGTCCAGTACTCCGACTTCAACGGCCCTGGCGAGAAATCCTGGCA  1019
```

```
D6   1020 GGCCCGCTACGACCTGAACATGGCCTCCTACGGCGTTCCCGGCCTGACCTTCATGGTTCG 1079
PAO1 1020 GGCTCGCTACGACCTGAACCTAGCCTCCTATGGCGTTCCCGGCCTGACTTTCATGGTCCG 1079

D6   1080 TTACATCAACGGTAAGGACATCGACGGCACCAAG--GTC-GACTCCA-GTTCCTCCTACG 1135
PAO1 1080 CTATATCAATGGCAAGGACATCGATGGCACCAAGATGTCTGACAACAACGTCGGC-TATA 1138

D6   1136 CGGGC--CTG-TACGGCGAGGACGGCAAGCACCACGAGACCAACCTGGAAGCCAAGTACG 1192
PAO1 1139 AGAACTACGGCTACGGCGAGGATGGCAAGCACCACGAAACCAACCTCGAAGCCAAGTACG 1198

D6   1193 TCGTCCAGGCCGGCCCGGCCAAGGACCTGTCGTTCCGTATCCGCCAGGCCTGGCACCGCG 1252
PAO1 1199 TGGTCCAGTCCGGTCCGGCCAAGGACCTGTCGTTCCGCATCCGCCAGGCCTGGCACCGTG 1258

D6   1253 CCAACGCCGACGAAGGTGAAGGCGACCAGAACGAGTTCCGCCTGATCGTCGACTATCCGC 1312
PAO1 1259 CCAACGCCGACCAGGGCGAAGGCGACCAGAACGAGTTCCGCCTGATCGTCGACTATCCGC 1320

D6   1313 TGTCGATCCTGTAA  1326
PAO1 1321 TGTCGATCCTGTAA  1332
```

Amino acid sequence

```
D6     MKVMKWSAIALAVSAGSTQFAVADAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSG   60
PAO1   MKVMKWSAIALAVSAGSTQFAVADAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSG   60

D6     DRVDWTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKTGTGNLPVMNDGKPRDDY   120
PAO1   DRVDWTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKTGTGNLPVMNDGKPRDDY   120

D6     SRAGGALKVRISKTMLKWGEMQPTAPVFAAGGSRLFPQTATGFQLQSSEFEGLDLEAGHF   180
PAO1   SRAGGAVKVRISKTMLKWGEMQPTAPVFAAGGSRLFPQTATGFQLQSSEFEGLDLEAGHF   180

D6     TEGKQGTTTKSRGELYATYAGQTAKSADFAGGRYAITDNLSASLYGAELKDIYRQYYLNT   240
PAO1   TEGKEPTTVKSRGELYATYAGETAKSADFIGGRYAITDNLSASLYGAELEDIYRQYYLNS   240

D6     NYTIPLASDQSLGFDFNIYRTTDEGKAKAGDISNTAWSLAGAYTLDAHTFTLAYQQVHGD   300
PAO1   NYTIPLASDQSLGFDFNIYRTNDEGKAKAGDISNTTWSLAAAYTLDAHTFTLAYQKVHGD   300

D6     EPFDYIGFGRNRSGGGGDSIFLANSVQYSDFNGPGEKSWQARYDLNMASYGVPGLTFMVR   360
PAO1   QPFDYIGFGRNGSGAGGDSIFLANSVQYSDFNGPGEKSWQARYDLNLASYGVPGLTFMVR   360

D6     YINGKDIDGTKVDSSSS-YAGL-YGEDGKHHETNLEAKYVVQAGPAKDLSFRIRQAWHRA   418
PAO1   YINGKDIDGTKMSDNNVGYKNYGYGEDGKHHETNLEAKYVVQSGPAKDLSFRIRQAWHRA   420

D6   NADEGEGDQNEFRLIVDYPLSIL  441
PAO1 NADQGEGDQNEFRLIVDYPLSIL  443
```

E1- OprD type

Nucleotide sequence

```
E1     1  ATGAAAGTGATGAAGTGGAGCGCCATTGCACTGGCGGTTTCCGCAGGTAGCACTCAGTTC  60
PAO1   1  ATGAAAGTGATGAAGTGGAGCGCCATTGCACTGGCGGTTTCCGCAGGTAGCACTCAGTTC  60

E1    61 GCCGTGGCCGACGCATTCGTCAGCGATCAGGCCGAAGCGAAGGGGTTCATCGAAGACAGC  120
PAO1  61 GCCGTGGCCGACGCATTCGTCAGCGATCAGGCCGAAGCGAAGGGGTTCATCGAAGACAGC  120

E1   121 AGCCTGAACCTGCTGCTCCGCAACTACTATTTCAACCGTGACGGCAAGGAAGGTC-GGGG  179
PAO1 121 AGCCTCGACCTGCTGCTCCGCAACTACTATTTCAACCGTGACGGCAAG-AGCGGCAGCGG  179

E1   180 TGATCGCGTCGATTGGACCCAGGGCTTCCTCACCACCTACGAATCCGGCTTCACTCAAGG  239
PAO1 180 GGACCGCGTCGACTGGACCCAAGGCTTCCTCACCACCTATGAATCCGGCTTCACCCAAGG  239

E1   240 CACCGTGGGCTTCGGCGTCGATGCCTTCGGCTACCTGGGCCTGAAGCTCGACGGCACCTC  299
PAO1 240 CACTGTGGGCTTCGGCGTCGATGCCTTCGGCTACCTGGGCCTGAAGCTCGACGGCACCTC  299
```

```
E1    300 CGACAAGACCGGCACCGGCAACCTGCCGGTGATGAACGACGGCAAGCCGCGCGATGACTA  359
PAO1  300 CGACAAGACCGGCACCGGCAACCTGCCGGTGATGAACGACGGCAAGCCGCGCGATGACTA  359

E1    360 CAGCCGCGCCGGCGGCGCCGTGAAGGTGCGCATCTCCAAGACCATGCTGAAGTGGGGCGA  419
PAO1  360 CAGCCGCGCCGGCGGCGCCGTGAAGGTGCGCATCTCCAAGACCATGCTGAAGTGGGGCGA  419

E1    420 GATGCAACCGACCGCCCCGGTCTTCGCCGCTGGCGGCAGCCGCCTGTTCCCGCAGACCGC  479
PAO1  420 GATGCAACCGACCGCCCCGGTCTTCGCCGCTGGCGGCAGCCGCCTGTTCCCGCAGACCGC  479

E1    480 GACCGGCTTCCAGCTGCAGAGCAGCGAATTCGAAGGGCTCGACCTCGAGGCAGGCCACTT  539
PAO1  480 GACCGGCTTCCAGCTGCAGAGCAGCGAATTCGAAGGGCTCGACCTCGAGGCAGGCCACTT  539

E1    540 CACCGAGGGCAAGGAGCCGACCACCGTCAAATCGCGCGGTGAGCTCTACGCCACCTACGC  599
PAO1  540 CACCGAGGGCAAGGAGCCGACCACCGTCAAATCGCGTGGCGAACTCTATGCCACCTACGC  599

E1    600 CGGCCAGACAGCCAAGAGCGCCGACTTCGCTGGCGGCCGCTACGCGATCACCGACCAACCT  659
PAO1  600 AGGCGAGACCGCCAAGAGCGCCGATTTCATTGGGGGCCGCTACGCAATCACCGATAACCT  659

E1    660 CAGCGCCTCCCTCTATGGCGCAGAGCTGAAAGACATCTATCGCCAGTAGTACCTGAACAC  719
PAO1  660 CAGCGCCTCCCTGTACGGCGCCGAACTCGAAGACATCTATCGCCAGTATTACCTGAACAG  719

E1    720 CAACTACACCATCCCGCTGGCCTCCGATCAATCGCTGGGCTTCGACTTCAACATCTACCG  779
PAO1  720 CAACTACACCATCCCACTGGCATCCGACCAATCGCTGGGCTTCGATTTCAACATCTACCG  779

E1    780 CACCACCGACGAAGGCAAGTCCAAGGCTGGCGACATCAGCAACACCACCTGGTCCCTGGC  839
PAO1  780 CACAAACGATGAAGGCAAGGCCAAGGCCGGCGACATCAGCAACACCACTTGGTCCCTGGC  839

E1    840 GGGCGCGTATACCTGGACGCCCACACCTTCACCCTGGCCTACCAGCAGGTGCATGGCGA  899
PAO1  840 GGCAGCCTACACTCTGGATGCGCACACTTTCACCTTGGCCTACCAGAAGGTCCATGGCGA  899

E1    900 CGAGCCGTTCGACTACATCGGCTTCGGCGGCAACGGTTCCGGCGCCGGCGGCGACTCGAT  959
PAO1  900 TCAGCCGTTTGATTATATCGGCTTCGGCCGCAACGGCTCTGGCGCAGGTGGCGACTCGAT  959

E1    960 CTTCCTCGCCAACTCCGTCCAGTACTCCGACTTCAACGGTCCTGGCGAGAAATCCTGGCA 1019
PAO1  960 TTTCCTCGCCAACTCTGTCCAGTACTCCGACTTCAACGGCCCTGGCGAGAAATCCTGGCA 1019

E1   1020GGCCCGCTACGACCTGAACCTGGCCTCCTACGGCGTTCCIGGCCTGACCTTCATGCTGCG 1079
PAO1 1020GGCTCGCTACGACCTGAACCTAGCCTCCTATGGCGTTCCCGGCCTGACTTTCATGGTCCG 1079

E1   1080TTACATCAATGGTAAGGACATCGACGGTACCAAG--GTC-GATTCCAGCTCCTCCTATGC 1136
PAO1 1080CTATATCAATGGCAAGGACATCGATGGCACCAAGATGTCTGACAACAACGTCGGCTATA- 1138

E1   1137AGGCCT--G--TACGGCGAGGATGGCAAGCACCACGAGACCAACCTCGAAGCCAAGTACG 1192
PAO1 1139AGAACTACGGCTACGGCGAGGATGGCAAGCACCACGAAACCAACCTCGAAGCCAAGTACG 1198

E1   1193TGGTCCAGTCCGGTCCGGCCAAGGACCTGTCGTTCCGCATCCGCCAGGCCTGGCACCGTG 1252
PAO1 1199TGGTCCAGTCCGGTCCGGCCAAGGACCTGTCGTTCCGCATCCGCCAGGCCTGGCACCGTG 1258

E1   1253CCAACGCCGACCAGGGCGAAGGCGACCAGAACGAGTTCCGCCTGATCGTCGACTATCCGC 1312
PAO1 1259CCAACGCCGACCAGGGCGAAGGCGACCAGAACGAGTTCCGCCTGATCGTCGACTATCCGC 1320

E1   1313TGTCGATCCTGTGA  1326
PAO1 1321TGTCGATCCTGTAA  1332
```

Amino acid sequence

```
E1      MKVMKWSAIALAVSAGSTQFAVADAFVSDQAEAKGFIEDSSLNLLLRNYYFNRDGKEGRG   60
PAO1    MKVMKWSAIALAVSAGSTQFAVADAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSG   60

E1      DRVDWTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKTGTGNLPVMNDGKPRDDY  120
PAO1    DRVDWTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKTGTGNLPVMNDGKPRDDY  120

E1      SRAGGAVKVRISKTMLKWGEMQPTAPVFAAGGSRLFPQTATGFQLQSSEFEGLDLEAGHF  180
PAO1    SRAGGAVKVRISKTMLKWGEMQPTAPVFAAGGSRLFPQTATGFQLQSSEFEGLDLEAGHF  180
```

```
E1      TEGKEPTTVKSRGELYATYAGQTAKSADFAGGRYAITDNLSASLYGAELKDIYRQ-----   235
PAO1    TEGKEPTTVKSRGELYATYAGETAKSADFIGGRYAITDNLSASLYGAELEDIYRQYYLNS   240
```

E2- OprD type

Nucleotide sequence

```
E2     1   ATGAAAGTGATGAAGTGGAGCGCCATTGCACTGGCGGTTTCCGCAGGTAGCACTCAGTTC   60
PAO1   1   ATGAAAGTGATGAAGTGGAGCGCCATTGCACTGGCGGTTTCCGCAGGTAGCACTCAGTTC   60

E2    61   GCCGTGGCCGACGCATTCGTCAGCGATCAGGCCGAAGCGAAGGGGTTCATCGAAGACAGC  120
PAO1  61   GCCGTGGCCGACGCATTCGTCAGCGATCAGGCCGAAGCGAAGGGGTTCATCGAAGACAGC  120

E2   121   AGCCTCGACCTGCTGCTCCGCAACTACTATTTCAACCGTGACGGCAAGAGCGGCAGCGGG  180
PAO1 121   AGCCTCGACCTGCTGCTCCGCAACTACTATTTCAACCGTGACGGCAAGAGCGGCAGCGGG  180

E2   181   GACCGCGTCGACTGGACCCAAGGCTTCCTCACCACCTATGAATCCGGCTTCACCCAAGGC  240
PAO1 181   GACCGCGTCGACTGGACCCAAGGCTTCCTCACCACCTATGAATCCGGCTTCACCCAAGGC  240

E2   241   ACCGTGGGCTTCGGCGTCGATGCCTTCGGCTACCTCGGCCTGAAGCTCGACGGCACCTCG  300
PAO1 241   ACTGTGGGCTTCGGCGTCGATGCCTTCGGCTACCTGGGCCTGAAGCTCGACGGCACCTCC  300

E2   301   GACAAGACCGGCACCGGCAACCTGCCGGTGATGAACGACGGCAAGCCGCGCGACGACTAC  360
PAO1 301   GACAAGACCGGCACCGGCAACCTGCCGGTGATGAACGACGGCAAGCCGCGCGATGACTAC  360

E2   361   AGCCGCGCTGGCGGCGCCCTGAAGGTGCGCATTTCCAAGACCATGCTGAAGTGGGGCGAA  420
PAO1 361   AGCCGCGCCGGCGGCGCCGTGAAGGTGCGCATCTCCAAGACCATGCTGAAGTGGGGCGAG  420

E2   421   ATGCAACCTACCGCGCCGGTCTTCGCCGCCGGCGGCAGCCGCCTGTTCCCGCAGACCGCG  480
PAO1 421   ATGCAACCGACCGCCCCGGTCTTCGCCGCTGGCGGCAGCCGCCTGTTCCCGCAGACCGCG  480

E2   481   ACCGGCTTCCAACTGCAGAGCAGTGAATTCGAAGGGCTCGATCTTGAAGCGGGCCACTTC  540
PAO1 481   ACCGGCTTCCAGCTGCAGAGCAGCGAATTCGAAGGGCTCGACCTCGAGGCAGGCCACTTC  540

E2   541   ACCGAAGGCAAGCAGGGCACCACCACCAAGTCGCGCGGCGAACTCTACGCAACCTATGCA  600
PAO1 541   ACCGAGGGCAAGGAGCCGACCACCGTCAAATCGCGTGGCGAACTCTATGCCACCTACGCA  600

E2   601   GGTCAGACCGCCAAGAGCGCGGACTTCGCCGGCGGCCGCTACGCGATCACCGACAATCTC  660
PAO1 601   GGCGAGACCGCCAAGAGCGCCGATTTCATTGGGGGCCGCTACGCAATCACCGATAACCTC  660

E2   661   AGCGCCTCCCTGTATGGCGCCGAATTGAAAGACATCTATCGCCAGTATTACCTGAACACC  720
PAO1 661   AGCGCCTCCCTGTACGGCGCCGAACTCGAAGACATCTATCGCCAGTATTACCTGAACAGC  720

E2   721   AACTACACCATCCCGCTGGCATCCGACCAGTCGCTGGGCTTCGACTTCAACATCTACCGC  780
PAO1 721   AACTACACCATCCCACTGGCATCCGACCAATCGCTGGGCTTCGATTTCAACATCTACCGC  780

E2   781   ACCACCGATGAAGGCAAAGCCAAGGCCGGCGACATCAGCAACACCGCCTGGTCCCTGGCC  840
PAO1 781   ACAAACGATGAAGGCAAGGCCAAGGCCGGCGACATCAGCAACACCACTTGGTCCCTGGC-  839

E2   841   GGC-GCGTACACCCTGGACGCGCACACCTTCACCCTGGCCTACCAGCAGGTGCATGGCGA  899
PAO1 840   GGCAGCCTACACTCTGGATGCGCACACTTTCACCTTGGCCTACCAGAAGGTCCATGGCGA  899

E2   900   CGAGCCGTTCGACTACATCGGCTTCGGCGAGAACCGTTCCGGCGGCGGCGGTGACTCGAT  959
PAO1 900   TCAGCCGTTTGATTATATCGGCTTCGGCCGCAACGGCTCTGGCGCAGGTGGCGACTCGAT  959

E2   960   TTTCCTCGCCAACTCCGTGCAGTACTCCGACTTCAACGGCCCCGGCGAGAAATCCTAGCA 1019
PAO1 960   TTTCCTCGCCAACTCTGTCCAGTACTCCGACTTCAACGGCCCTGGCGAGAAATCCTGGCA 1019

E2   1020  GGCCCGCTACGACCTGAACATGGCCTCCTACGGCGTTCCCGGCCTGACCTTCATGGTTCG 1079
PAO1 1020  GGCTCGCTACGACCTGAACCTAGCCTCCTATGGCGTTCCCGGCCTGACTTTCATGGTCCG 1079
```

```
E2    1080 TTACATCAACGGTAAGGACATCGACGGCACCAAG--GTC-GACTCCA-GTTCCTCCTACG 1135
PAO1  1080 CTATATCAATGGCAAGGACATCGATGGCACCAAGATGTCTGACAACAACGTCGGC-TATA 1138

E2    1136 CGGGC--CTG-TACGGCGAGGACGGCAAGCACCACGAGACCAACCTGGAAGCCAAGTACG 1192
PAO1  1139 AGAACTACGGCTACGGCGAGGATGGCAAGCACCACGAAACCAACCTCGAAGCCAAGTACG 1198

E2    1193 TCGTCCAGGCCGGCCCGGCCAAGGACCTGTCGTTCCGTATCCGCCAGGCCTGGCACCGCG 1252
PAO1  1199 TGGTCCAGTCCGGTCCGGCCAAGGACCTGTCGTTCCGCATCCGCCAGGCCTGGCACCGTG 1258

E2    1253 CCAACGCCGACGAAGGTGAAGGCGACCAGAACGAGTTCCGCCTGATCGTCGACTATCCGC 1312
PAO1  1259 CCAACGCCGACCAGGGCGAAGGCGACCAGAACGAGTTCCGCCTGATCGTCGACTATCCGC 1320

E2    1313 TGTCGATCCTGTAA  1326
PAO1  1321 TGTCGATCCTGTAA  1332
```

Amino acid sequence

```
E2    MKVMKWSAIALAVSAGSTQFAVADAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSG  60
PAO1  MKVMKWSAIALAVSAGSTQFAVADAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSG  60

E2    DRVDWTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKTGTGNLPVMNDGKPRDDY  120
PAO1  DRVDWTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKTGTGNLPVMNDGKPRDDY  120

E2    SRAGGALKVRISKTMLKWGEMQPTAPVFAAGGSRLFPQTATGFQLQSSEFEGLDLEAGHF  180
PAO1  SRAGGAVKVRISKTMLKWGEMQPTAPVFAAGGSRLFPQTATGFQLQSSEFEGLDLEAGHF  180

E2    TEGKQGTTTKSRGELYATYAGQTAKSADFAGGRYAITDNLSASLYGAELKDIYRQYYLNT  240
PAO1  TEGKEPTTVKSRGELYATYAGETAKSADFIGGRYAITDNLSASLYGAELEDIYRQYYLNS  240

E2    NYTIPLASDQSLGFDFNIYRTTDEGKAKAGDISNTAWSLAGAYTLDAHTFTLAYQQVHGD  300
PAO1  NYTIPLASDQSLGFDFNIYRTNDEGKAKAGDISNTTWSLAAAYTLDAHTFTLAYQKVHGD  300

E2    EPFDYIGFGENRSGGGDSIFLANSVQYSDFNGPGEKS----------------------  338
PAO1  QPFDYIGFGRNGSGAGGDSIFLANSVQYSDFNGPGEKSWQARYDLNLASYGVPGLTFMVR  360
```

F- OprD type

Nucleotide sequence

```
F     1  ATGAAAGTGATGAAGTGGAGCGCCATTGCACTGGCGGTTTCCGCAGGTAGCACTCAGTTC  60
PAO1  1  ATGAAAGTGATGAAGTGGAGCGCCATTGCACTGGCGGTTTCCGCAGGTAGCACTCAGTTC  60

F     61  GCCGTGGCCGACGCATTCGTCAGCGATCAGGCCGAAGCGAAGGGGTTCATCGAAGACAGC  120
PAO1  61  GCCGTGGCCGACGCATTCGTCAGCGATCAGGCCGAAGCGAAGGGGTTCATCGAAGACAGC  120

F     121 AGCCTCGACCTGCTGCTCCGCAACTACTATTTCAACCGTGACGGCAAGAGCGGCAGCGGG  180
PAO1  121 AGCCTCGACCTGCTGCTCCGCAACTACTATTTCAACCGTGACGGCAAGAGCGGCAGCGGG  180

F     181 GACCGCGTCGACTGGACCCAAGGCTTCCTCACCACCTATGAATCCGGCTTCACCCAAGGC  240
PAO1  181 GACCGCGTCGACTGGACCCAAGGCTTCCTCACCACCTATGAATCCGGCTTCACCCAAGGC  240

F     241 ACTGTGGGCTTCGGCGTCGATGCCTTCGGCTACCTGGGCCTGAAGCTCGACGGCACCTCC  300
PAO1  241 ACTGTGGGCTTCGGCGTCGATGCCTTCGGCTACCTGGGCCTGAAGCTCGACGGCACCTCC  300

F     301 GACAAGACCGGCACCGGCAACCTGCCGGTGATGAACGACGGCAAGCCGCGCGATGACTAC  360
PAO1  301 GACAAGACCGGCACCGGCAACCTGCCGGTGATGAACGACGGCAAGCCGCGCGATGACTAC  360

F     361 AGCCGCGCCGGCGGCGCCGTGAAGGTGCGCATCTCCAAGACCATGCTGAAGTGGGGCGAG  420
PAO1  361 AGCCGCGCCGGCGGCGCCGTGAAGGTGCGCATCTCCAAGACCATGCTGAAGTGGGGCGAG  420

F     421 ATGCAACCGACCGCCCCGGTCTTCGCCGCTGGCGGCAGCCGCCTGTTCCCGCAGACCGCG  480
PAO1  421 ATGCAACCGACCGCCCCGGTCTTCGCCGCTGGCGGCAGCCGCCTGTTCCCGCAGACCGCG  480

F     481 ACCGGCTTCCAGCTGCAGAGCAGCGAATTCTAAGGGCTCGACCTCGAGGCAGGCCACTTC  540
PAO1  481 ACCGGCTTCCAGCTGCAGAGCAGCGAATTCGAAGGGCTCGACCTCGAGGCAGGCCACTTC  540
```

```
F     541 ACCGAGGGCAAGGAGCCGACCACCGTCAAATCGCGTGGCGAACTCTATGCCACCTACGCA  600
PAO1  541 ACCGAGGGCAAGGAGCCGACCACCGTCAAATCGCGTGGCGAACTCTATGCCACCTACGCA  600

F     601 GGCGAGACCGCCAAGAGCGCCGATTTCATTGGGGGCCGCTACGCAATCACCGATAACCTC  660
PAO1  601 GGCGAGACCGCCAAGAGCGCCGATTTCATTGGGGGCCGCTACGCAATCACCGATAACCTC  660

F     661 AGCGCCTCCCTGTACGGCGCCGAACTCGAAGACATCTATCGCCAGTATTACCTGAACAGC  720
PAO1  661 AGCGCCTCCCTGTACGGCGCCGAACTCGAAGACATCTATCGCCAGTATTACCTGAACAGC  720

F     721 AACTACACCATCCCACTGGCATCCGACCAATCGCTGGGCTTCGATTTCAACATCTACCGC  780
PAO1  721 AACTACACCATCCCACTGGCATCCGACCAATCGCTGGGCTTCGATTTCAACATCTACCGC  780

F     781 ACAAACGATGAAGGCAAGGCCAAGGCCGGCGACATCAGCAACACCACTTGGTCCCTGGCG  840
PAO1  781 ACAAACGATGAAGGCAAGGCCAAGGCCGGCGACATCAGCAACACCACTTGGTCCCTGGCG  840

F     841 GCAGCCTACACTCTGGATGCGCACACTTTCACCTTGGCCTACCAGAAGGTCCATGGCGAT  900
PAO1  841 GCAGCCTACACTCTGGATGCGCACACTTTCACCTTGGCCTACCAGAAGGTCCATGGCGAT  900

F      901 CAGCCGTTTGATTATATCGGCTTCGGCCGCAACGGCTCTGGCGCAGGTGGCGACTCGATT  960
PAO1   901 CAGCCGTTTGATTATATCGGCTTCGGCCGCAACGGCTCTGGCGCAGGTGGCGACTCGATT  960

F      961 TTCCTCGCCAACTCTGTCCAGTACTCCGACTTCAACGGCCCTGGCGAGAAATCCTGGCAG 1020
PAO1   961 TTCCTCGCCAACTCTGTCCAGTACTCCGACTTCAACGGCCCTGGCGAGAAATCCTGGCAG 1020

F     1021 GCTCGCTACGACCTGAACCTAGCCTCCTATGGCGTTCCCGGCCTGACTTTCATGGTCCGC 1080
PAO1  1021 GCTCGCTACGACCTGAACCTAGCCTCCTATGGCGTTCCCGGCCTGACTTTCATGGTCCGC 1080

F     1081 TATATCAATGGCAAGGACATCGATGGCACCAAGATGTCTGACAACAACGTCGGCTATAAG 1140
PAO1  1081 TATATCAATGGCAAGGACATCGATGGCACCAAGATGTCTGACAACAACGTCGGCTATAAG 1140

F     1141 AACTACGGCTACGGCGAGGACGGCAAGCACCACGAGACCAACCTCGAAGCCAAGTACGTG 1200
PAO1  1141 AACTACGGCTACGGCGAGGATGGCAAGCACCACGAAACCAACCTCGAAGCCAAGTACGTG 1200

F     1201 GTCCAGTCCGGTCCGGCCAAGGACCTGTCGTTCCGCATCCGCCAGGCCTGGCACCGTGCC 1260
PAO1  1201 GTCCAGTCCGGTCCGGCCAAGGACCTGTCGTTCCGCATCCGCCAGGCCTGGCACCGTGCC 1260

F     1261 AACGCCGACCAGGGCGAAGGCGACCAGAACGAGTTCCGCCTGATCGTCGACTATCCGCTG 1320
PAO1  1261 AACGCCGACCAGGGCGAAGGCGACCAGAACGAGTTCCGCCTGATCGTCGACTATCCGCTG 1320

F     1321 TCGATCCTGTAA  1332
PAO1  1321 TCGATCCTGTAA  1332
```

Amino acid sequence

```
F        MKVMKWSAIALAVSAGSTQFAVADAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSG    60
PAO1     MKVMKWSAIALAVSAGSTQFAVADAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSG    60

F        DRVDWTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKTGTGNLPVMNDGKPRDDY    120
PAO1     DRVDWTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKTGTGNLPVMNDGKPRDDY    120

F        SRAGGAVKVRISKTMLKWGEMQPTAPVFAAGGSRLFPQTATGFQLQSSEF----------    170
PAO1     SRAGGAVKVRISKTMLKWGEMQPTAPVFAAGGSRLFPQTATGFQLQSSEFEGLDLEAGHF    180
```

* *Point mutations are highlighted in magenta, deletions in cyan, insertions in silver and amino acid changes in green colour. Nucleotide sequences highlighted in yellow denote additional point mutations (silent) shown by individual isolates Pa20 and Pa22 (C2- oprD type), Pa14 (D3- OprD type) and Pa23 (D5-OprD type). Nucleotide sequences highlighted in olive denote additional point mutations (silent) shown by individual isolates Pa12 (C2-oprD type) and Pa17,Pa14, Pa24, Pa28 (D3- OprD type). Nucleotide sequence in black denotes additional point mutation (silent) shown by Pa7 (D3-OprD type) and in blue by*

Pa14, Pa24 and Pa28 (D3-OprD type). Nucleotide sequences highlighted in red colour denote premature stop codons.

***The loop regions as determined by the crystal structure of the porin protein have been superimposed as a blue coloured line over the amino acid sequences in A-OprD type (Kos et al., 2016).*

Sequences of *oprD* gene derived from amplicons of 26 isolates with down regulated porins were subjected to Bayesian phylogenetic analysis along with a select group of nine closely related sequences of *P. aeruginosa* isolates deposited in the NCBI database including the reference strain *P. aeruginosa* strain, PAO1 (GenBank accession no. AE004091.2). *P. citronellosi* (GenBank accession no. CP014158.1) was taken as the outgroup for the purpose. The sequences were chosen based on geographical location of microbial collection, highest query coverage and minimum 'E' values which were then subjected to BLASTN analysis with the sequences from PCR amplicons obtained in this study. The best fit model of sequence evolution based on AIC was detected as GTR+I+G. PSRF value was observed to be 1.0 and the ESS was above 100. Analysis was run for 3,000,000 generations and clade credibility values shown at each node were found to be >0.5. Phylogenetic analysis (Fig. 4.21) revealed that isolates categorized as A-OprD type identical to wild type such as Pa2, Pa6, Pa15 and Pa18 along with Pa29 of B2-OprD type with single amino acid change were found to exhibit similarity with PAO1 reference strain and with another strain reported from France (GenBank accession no. LT673656.1). OprD types with several polymorphisms, Pa4 (C1 type) showed similarity with the isolate from Brazil (GenBank accession no. CP021380.1), Pa13 (C3 type) and Pa21 (C4 type) similar to that from Taiwan (GenBank accession no. CP004061.1) whilst Pa20 and Pa22 (C2 type) bore similarity to those from Singapore (GenBank accession no. CP020704.1). Pa23 which belonged to D5-OprD type displayed several polymorphisms and sequence divergence between amino acid position 372 (VDSSSS-YAGL-) to 383 was found to be similar to the *P.aeruginosa* isolate from North America (GenBank accession no. CP012901.1); Pa25 isolate (E1 type) with truncated porin protein displayed similarity with those isolated from Malaysia (GenBank accession no. CP007147.1).

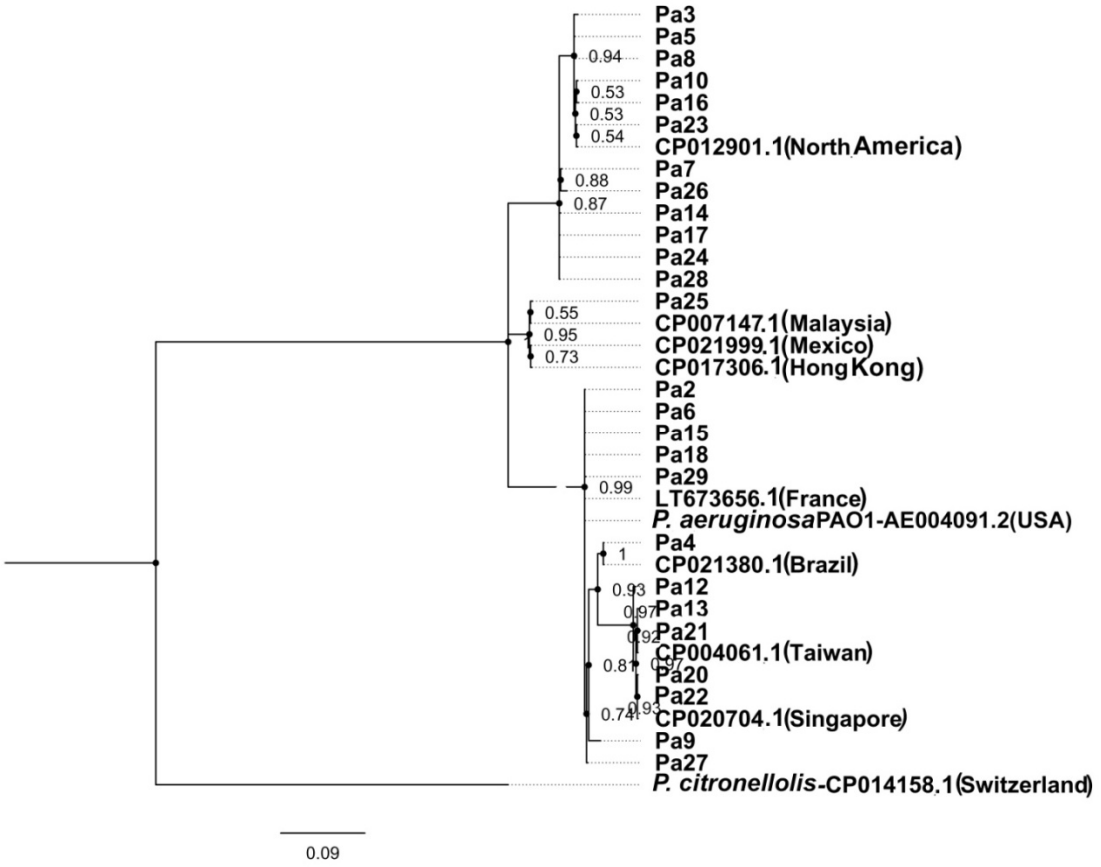


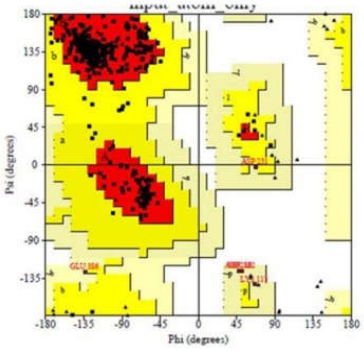
Fig. 4.21. Bayesian phylogenetic tree reconstructed from employing oprD ampliconic sequences from P.aeruginosa isolates. *Scale bar denotes probability of nucleotide change. Analysis was run for 3,000,000 generations. Clade credibility values are shown at each node. P. citronellolis (GenBank accession no. CP014158.1) was used as the outgroup.*

4.4.9 Molecular modelling of OprD protein

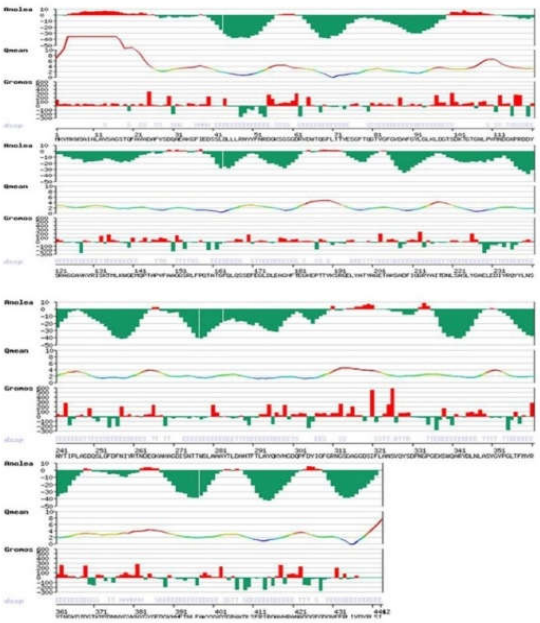
The outer membrane porin protein OprD of *P.aeruginosa,* which facilitates the uptake of carbapenem antibiotics, consists of 443 amino acids. Crystal structure of the protein revealed the presence of a monomeric 18-strand transmembrane beta-barrel comprising 9 loops. The protein is thought to assemble into a trimeric form due to the occurrence of two shorter beta-strands S5 and S6 (Biswas et al., 2007). Using a homology modelling approach, 3D models of the OprD were built and validated (Figs. 4.22-4.26) deploying the bioinformatics tools mentioned in section 3.15 (Materials and Methods). The mutation-induced structural variations in four selected mutants Pa12, Pa7, Pa25 and Pa27 of different OprD types were compared

with the wild type OprD of the reference strain PAO1(Figs. 4.27-4.30). Pa12 represented C2-OprD type mutants harbouring several amino acid polymorphisms whilst Pa7 symbolized mutants displaying typical deletions-substitutions such as $_{372}$(VDSSSS -YAGL-)$_{383}$ [Table 4.15 and 4.16] in addition to amino acid polymorphisms. The remaining two Pa25 and Pa27 were illustrative of mutants bearing protein truncations at different regions. For comparative modelling the crystal structure of A-chain of OprD (PDB ID: 4FOZ) was identified as the best template. The query coverage with target was 94% and the E value was 0.0. Identity and similarity percentage was 99% each and the alignment score was found to be 852. Mutations in the modelled proteins of Pa12 were confined to L2, b/w L3 and L4, L4, L7 loops and those of Pa7 were located in L1, b/w L2 and L3, L4, b/w L4 and L5, L5, b/w L5 and L6, L6, b/w L6 and L7, L7, b/w L7 and L8, L8, b/w L8 and L9, L9 loops (Kos et al., 2016). Structurally the modelled mutant proteins of Pa12 and Pa7, however, cannot be visually differentiated from that of the wild type, PAO1. The structure of the truncated mutant proteins from Pa25and Pa27 is strikingly conspicuous and discernible visually due the loss of substantial portions of the polypeptide.

a)



b)



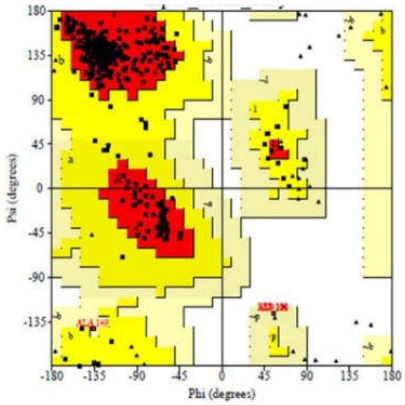
c)

Scoring function term	Raw score	Z-score
C_beta interaction energy	32.46	-2.98
All-atom pairwise energy	-3366.02	-2.68
Solvation energy	23.44	-5.37
Torsion angle energy	-46.15	-3.14
Secondary structure agreement	70.9%	-1.95
Solvent accessibility agreement	61.4%	-3.69
QMEAN6 score	0.383	-4.53

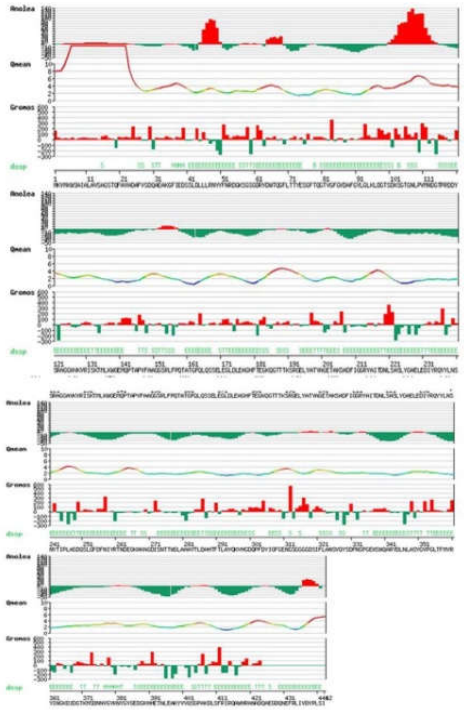
Fig. 4.22. Refinement and validation of modelled OprD (reference-P. aeruginosa PAO1).

a) Stereo chemical validation by Ramachandran plot indicted that 90.4% residues are in most favoured region (A, B, L) and 8.2% residues are in additional allowed region [a, b, l, p]. b) Energy minimization of the modelled protein was refined and validated by ANOLEA, QMEAN, GROMOS and secondary structure by DSSP. c) Energy Features of the final model.

a)



b)



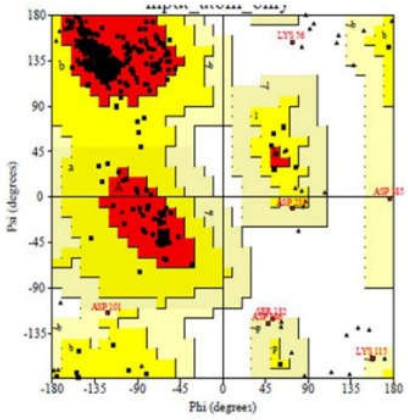
c)

Scoring function term	Raw score	Z-score
C_beta interaction energy	14.90	-2.66
All-atom pairwise energy	-3323.32	-2.73
Solvation energy	21.39	-5.20
Torsion angle energy	-54.92	-2.78
Secondary structure agreement	72.0%	-1.71
Solvent accessibility agreement	60.9%	-3.78
QMEAN6 score	0.388	-4.47

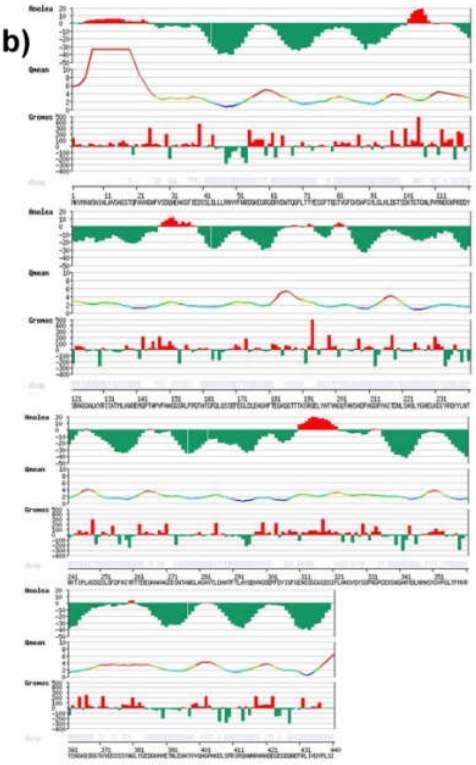
Fig. 4.23. Refinement and validation of modelled OprD (P. aeruginosa mutant-Pa12).

a)Stereo chemical validation by Ramachandran plot indicted that 90.4% residues are in most favoured region (A, B, L) and 8.8% residues are in additional allowed region [a, b, l, p]. b) Energy minimization of the modelled protein was refined and validated by ANOLEA, QMEAN, GROMOS and secondary structure by DSSP. c) Energy Features of the final model.

a)



b)



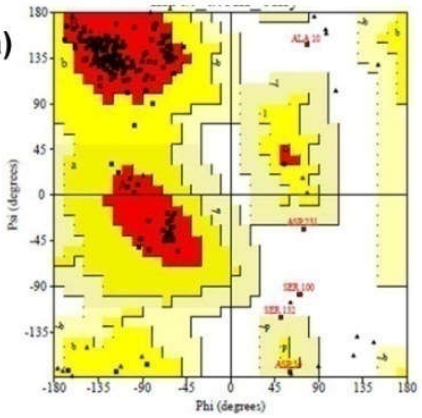
c)

Scoring function term	Raw score	Z-score
C_beta interaction energy	15.44	-2.72
All-atom pair wise energy	-3577.31	-2.61
Solvation energy	22.21	-5.31
Torsion angle energy	-45.23	-3.17
Secondary structure agreement	70.3%	-2.08
Solvent accessibility agreement	63.9%	-3.22
QMEAN6 score	0.418	-4.13

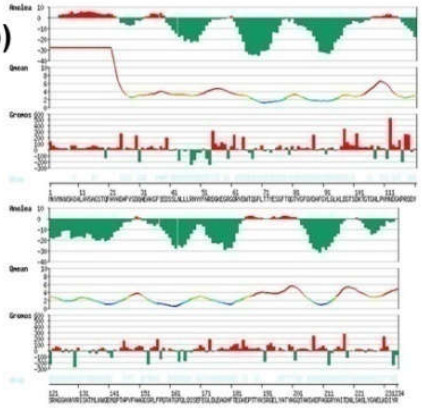
Fig. 4.24. Refinement and validation of modelled OprD (P. aeruginosa mutant-Pa7).

a) Stereo chemical validation by Ramachandran plot indicted that 90.6% residues are in most favoured region (A, B, L) and 7.5% residues are in additional allowed region [a, b, l, p]. b) Energy minimization of the modelled protein was refined and validated by ANOLEA, QMEAN, GROMOS and secondary structure by DSSP. c) Energy Features of the final model.

a)



b)

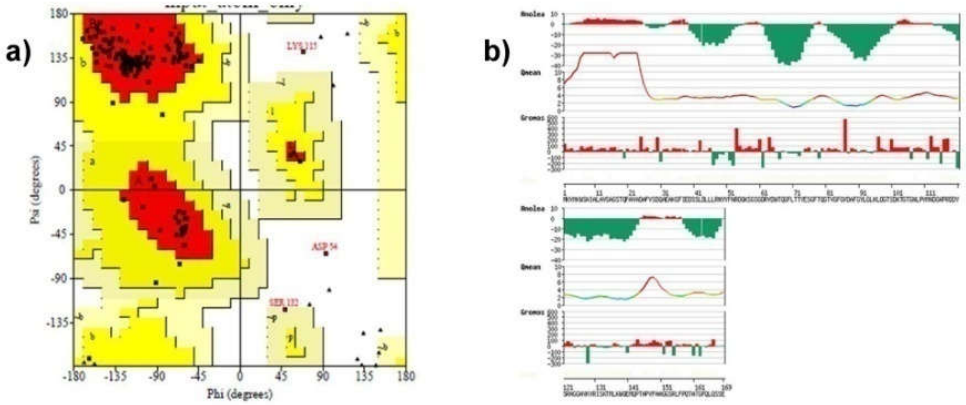


c)

Scoring function term	Raw score	Z-score
C_beta interaction energy	21.79	-4.09
All-atom pairwise energy	-935.54	-3.23
Solvation energy	18.04	-4.98
Torsion angle energy	-38.86	-1.67
Secondary structure agreement	68.1%	-2.43
Solvent accessibility agreement	55.7%	-4.42
QMEAN6 score	0.322	-4.85

Fig. 4.25. Refinement and validation of modelled OprD (P. aeruginosa mutant-Pa25).

a) Stereo chemical validation by Ramachandran plot indicted that 92.9% residues are in most favoured region (A, B, L) and 4.6% residues are in additional allowed region [a, b, l, p]. b) Energy minimization of the modelled protein was refined and validated by ANOLEA, QMEAN, GROMOS and secondary structure by DSSP. c) Energy Features of the final model.



c)

Scoring function term	Raw score	Z-score
C_beta interaction energy	30.01	-4.88
All-atom pairwise energy	254.65	-4.03
Solvation energy	17.56	-4.67
Torsion angle energy	-29.21	-1.43
Secondary structure agreement	65.3%	-2.50
Solvent accessibility agreement	50.6%	-4.71
QMEAN6 score	0.235	-5.35

Fig. 4.26. Refinement and validation of modelled OprD (P. aeruginosa mutant-Pa27).

a) Stereo chemical validation by Ramachandran plot indicted that 91.5% residues are in most favoured region (A, B, L) and 6.4% residues are in additional allowed region [a, b, l, p]. b) Energy minimization of the modelled protein was refined and validated by ANOLEA, QMEAN, GROMOS and secondary structure by DSSP. c) Energy Features of the final model.

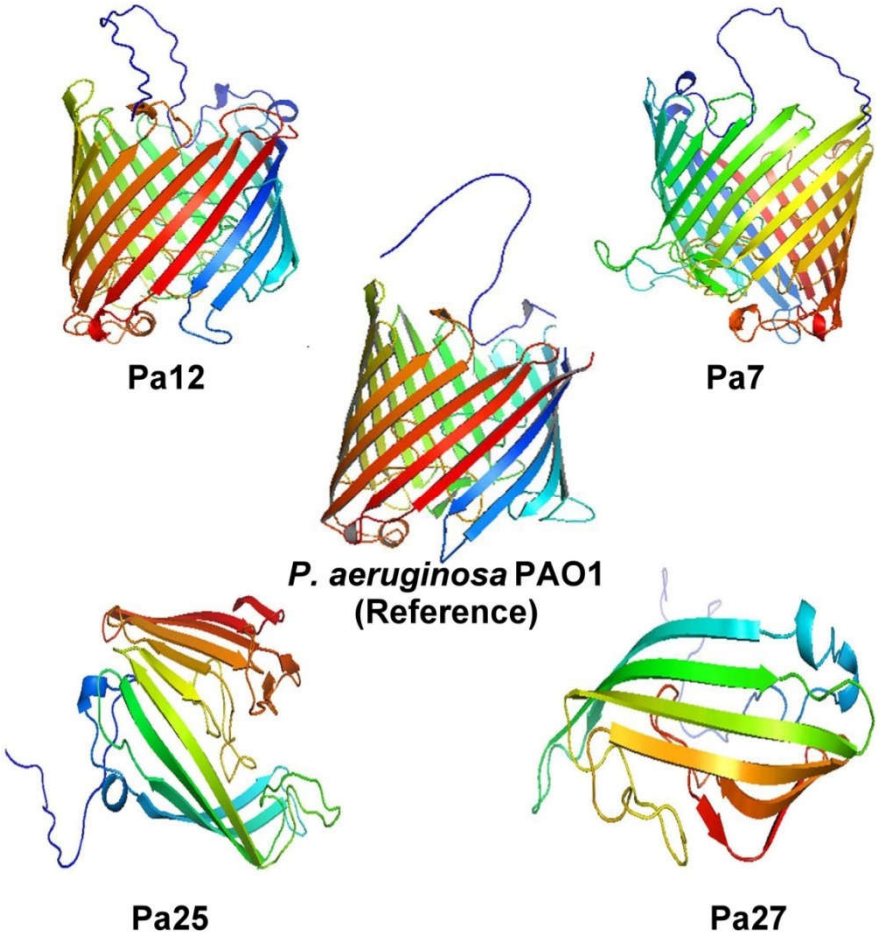
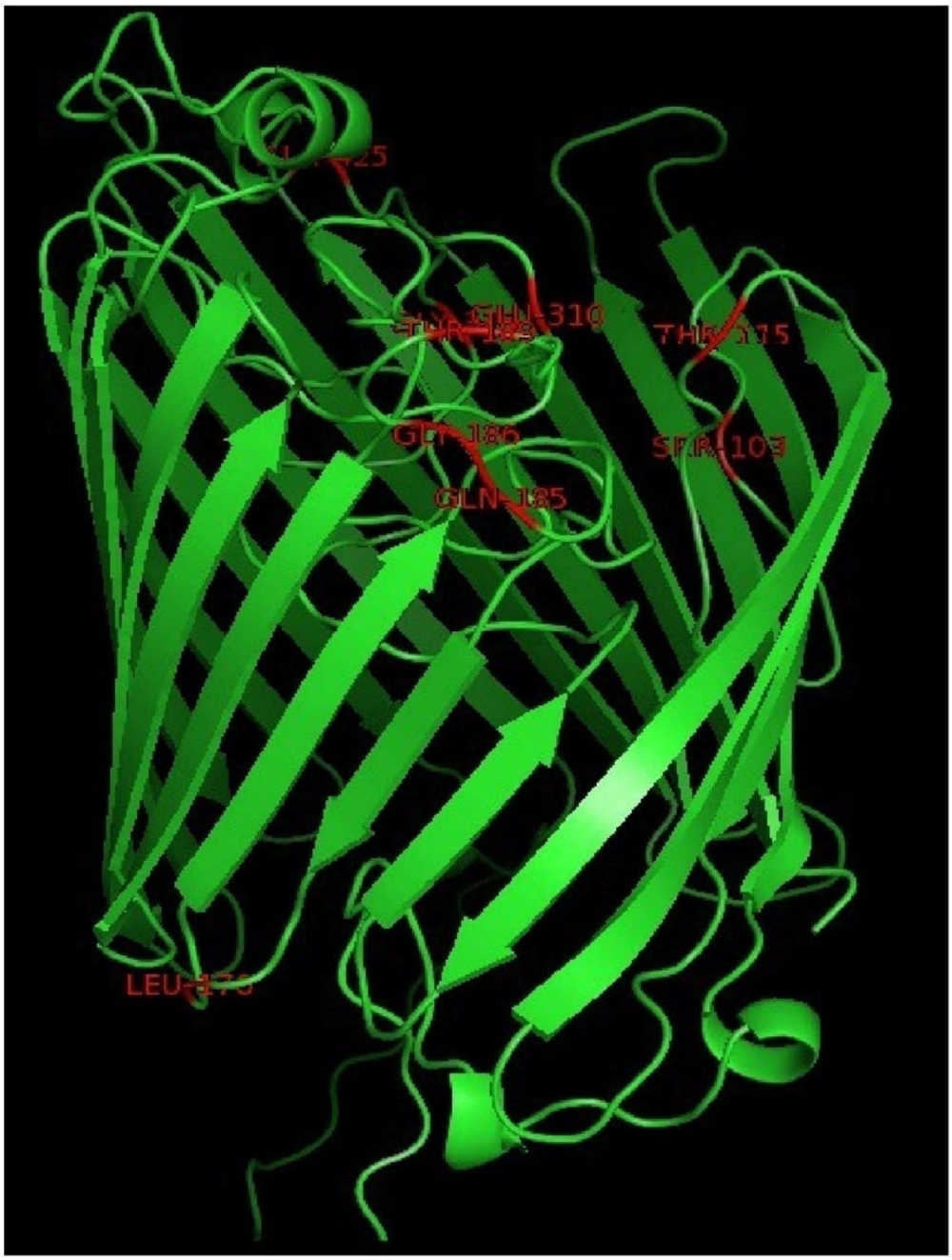


Fig. 4.27. 3D model of porin OprD of P. aeruginosa PAO1 wild type strain (reference)and four mutants, Pa12, Pa7, Pa25 and Pa27. *Pa12 mutant harbours several amino acid polymorphisms; Pa7 displays the typical* $_{372}$*(VDSSSS-YAGL-)*$_{383}$ *amino acid deletions-substitutions in addition to amino acid polymorphisms; Pa25 mutant truncated protein along with several amino acid changes; Pa27 mutant truncated protein.*

a)



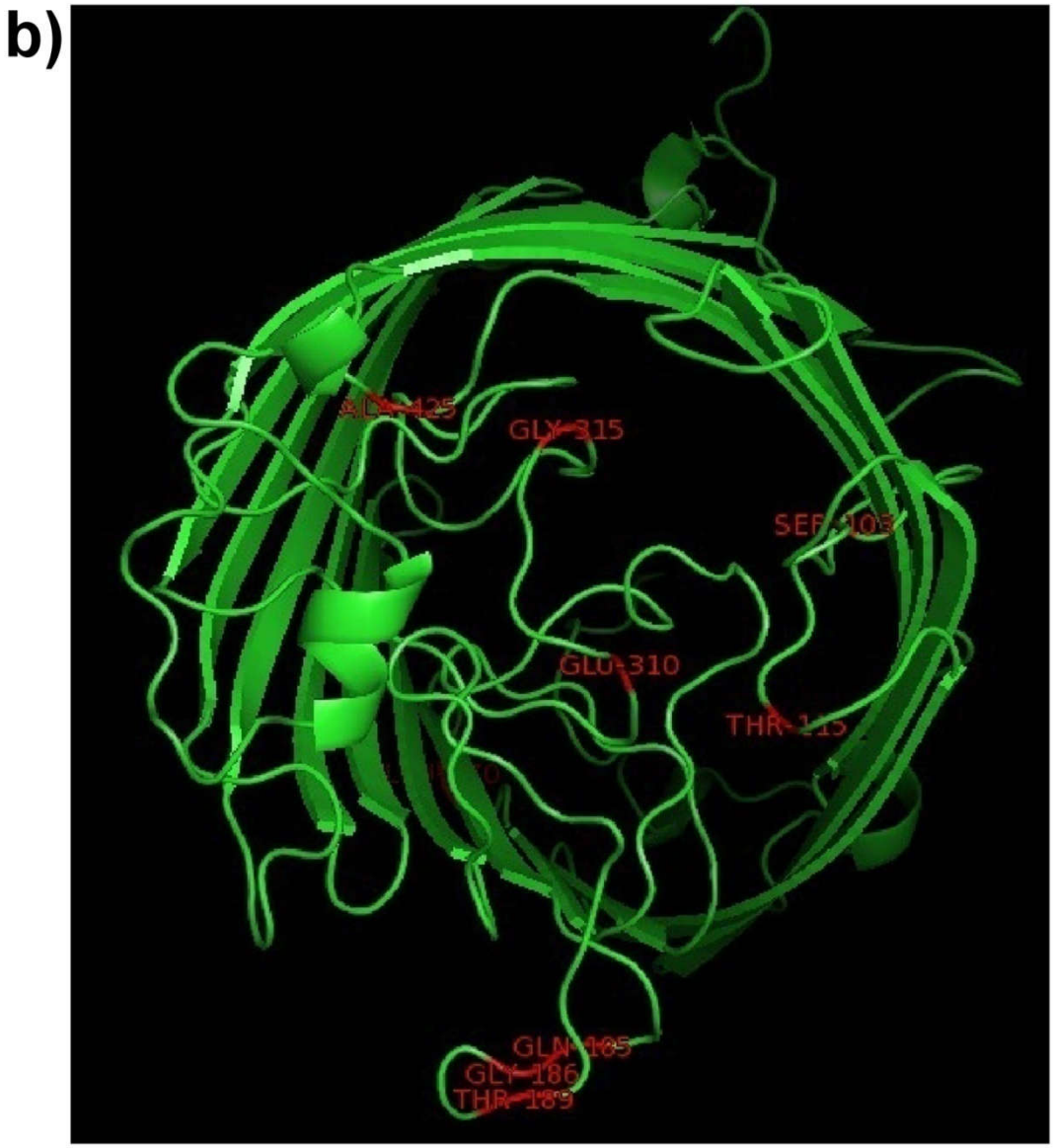


Fig. 4.28. Enlarged view of the molecular model of Pa12 OprD protein*. a) Side view; b) Top view- looking through the channel. Mutations are depicted in red colour, labeled with capitalized three-letter amino acid codes and their position (SER-Serine, THR-Threonine, LEU-Leucine, GLN-Glutamine, GLY-Glycine, GLU-Glutamic acid and ALA-Alanine).*

a)

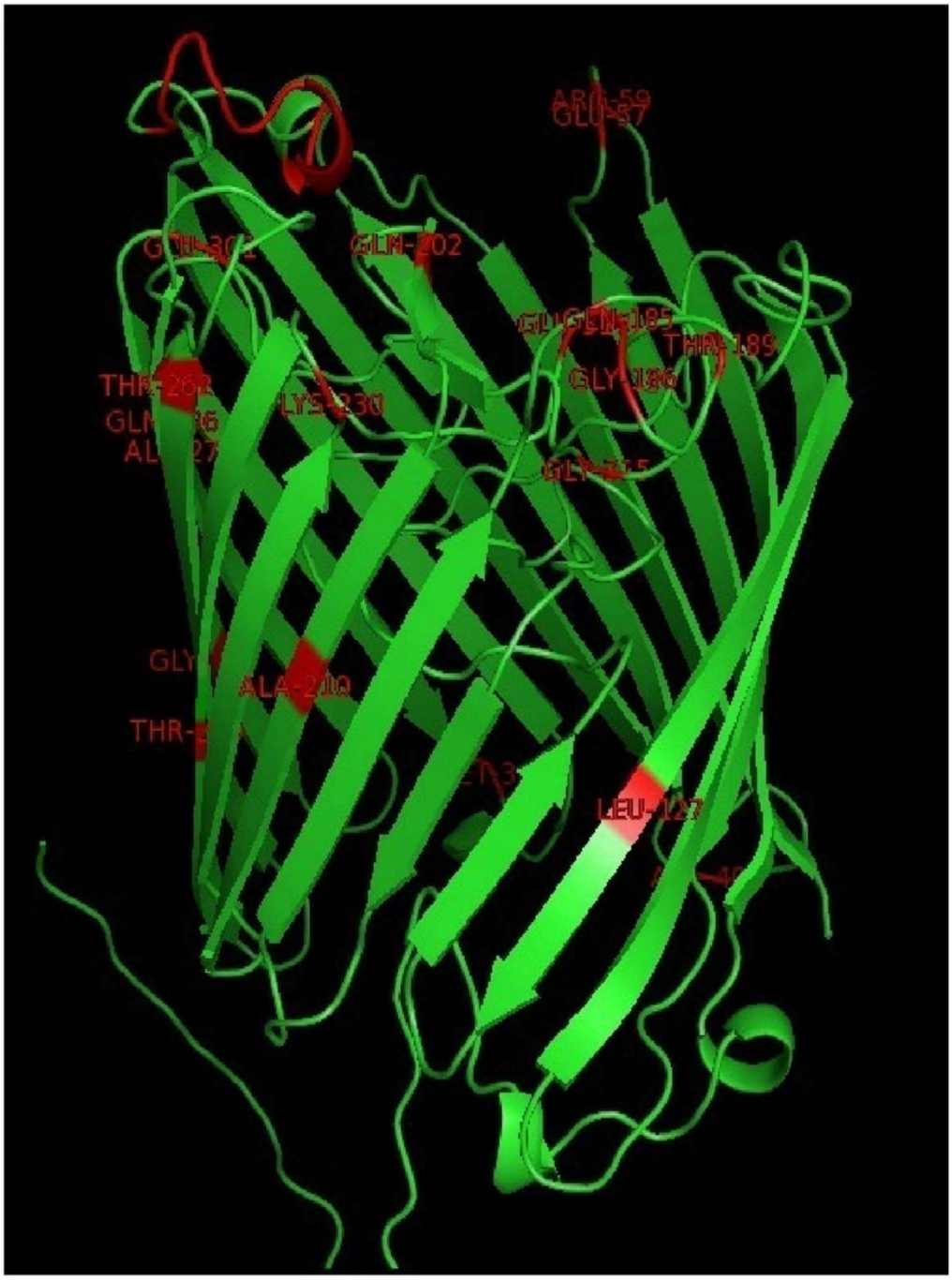
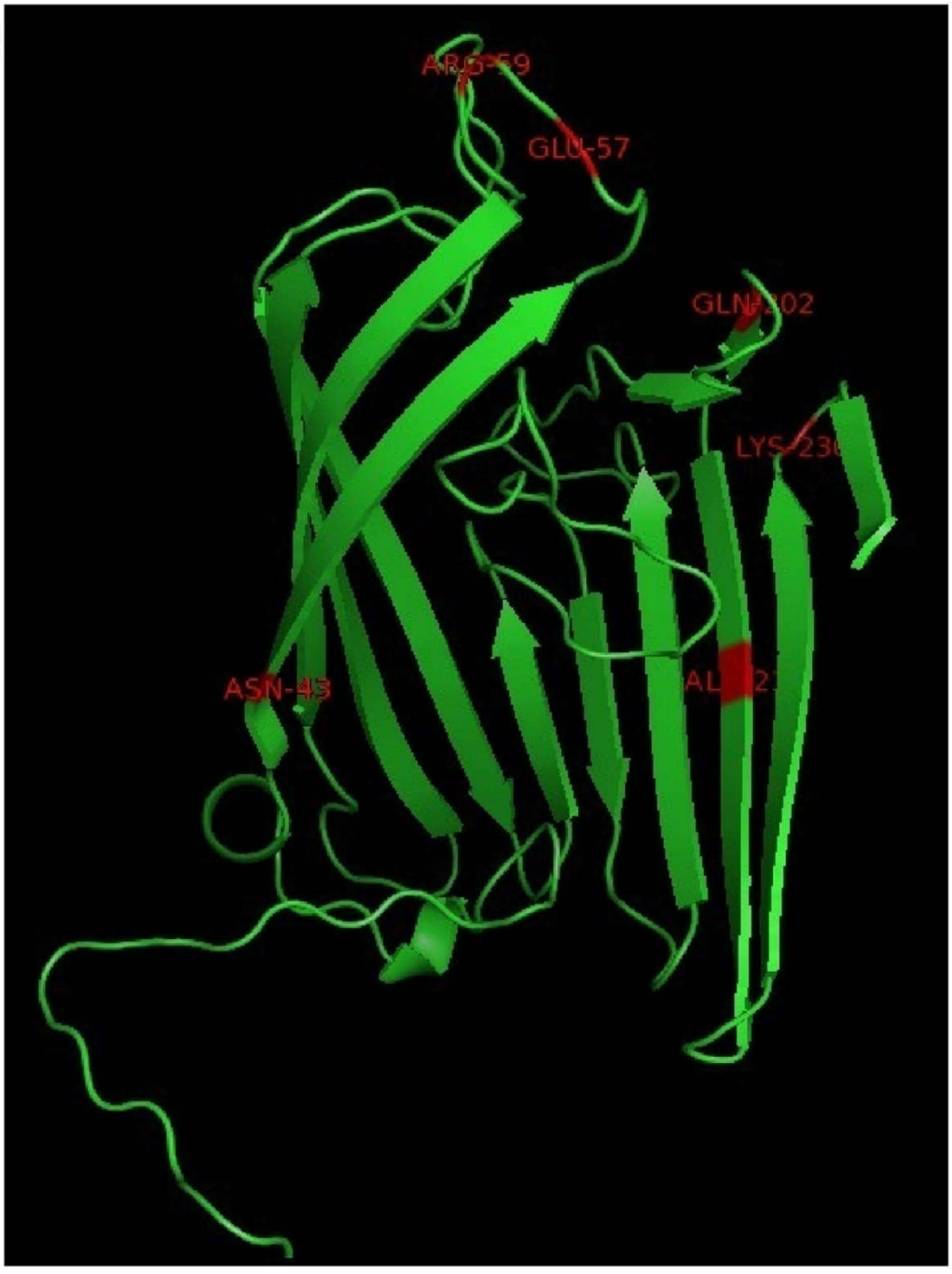


Fig. 4.29. Enlarged view of the molecular model of Pa7 OprD protein. . *a) Side view of OprD; b) Top view- looking through the channel. Mutations are depicted in red colour, labeled with capitalized three-letter amino acid codes and their position (ARG-Arginine, GLU-Glutamic acid, ALA-Alanine, GLN-Glutamine, THR-Threonine, GLY-Glycine, LEU-Leucine, LYS-Lysine and MET-Methionine).*

a)



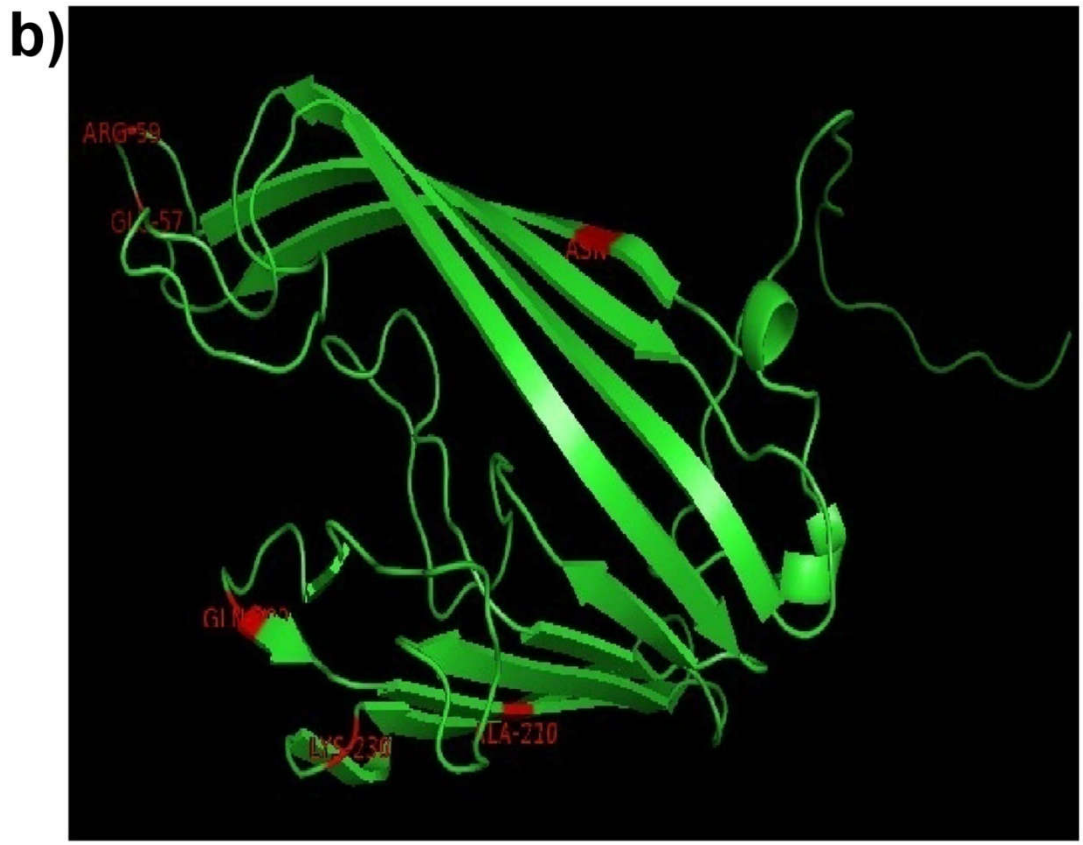


Fig. 4.30. Enlarged view of the molecular model of Pa25 truncated OprD protein. *(a) and (b) represent two different views. Mutations are depicted in red colour, labeled with capitalized three-letter amino acid codes and their position (ASN-Asparagine, GLU-Glutamic acid, ARG-Arginine, GLN-Glutamine, ALA-Alanine and LYS-Lysine).*

4.4.10 Restriction mapping of *oprD* gene

The amplicons of *oprD* genes obtained from the mutants were found to be about 1500 bp (section 4.4.8, Fig. 4.20) in size as expected. The gene length for reference strain PAO1 has been reported as 1332 bp (Winsor et al., 2016). In an attempt to further characterize the DNA fragment, the *oprD* sequence of the reference PAO1 strain retrieved from GenBank and the mutant sequences obtained in the study were together subjected to a scan with the NEB cutter V2.0 (Vincze et al., 2003) to detect the presence of restriction sites therein. Reproducible restriction patterns were obtained with restriction endonucleases, *Hinc* II, *Pst* I and *Pvu* II. With respect to the wild type porin gene of PAO1, *Hinc* II enzyme was found to restrict the cognate sequence 5'GT(C/T)↓(A/G)AC3' at 189th and 1308th position whilst *Pst* I restricted the sequence 5'CTGCA↓G3' at 497th position and *Pvu* II at 492nd position of the recognition sequence 5'CAG↓CTG3'. The restriction profiles are shown in Figs 4.31-4.33 and the details of the restriction analysis are given in Table 4.17.

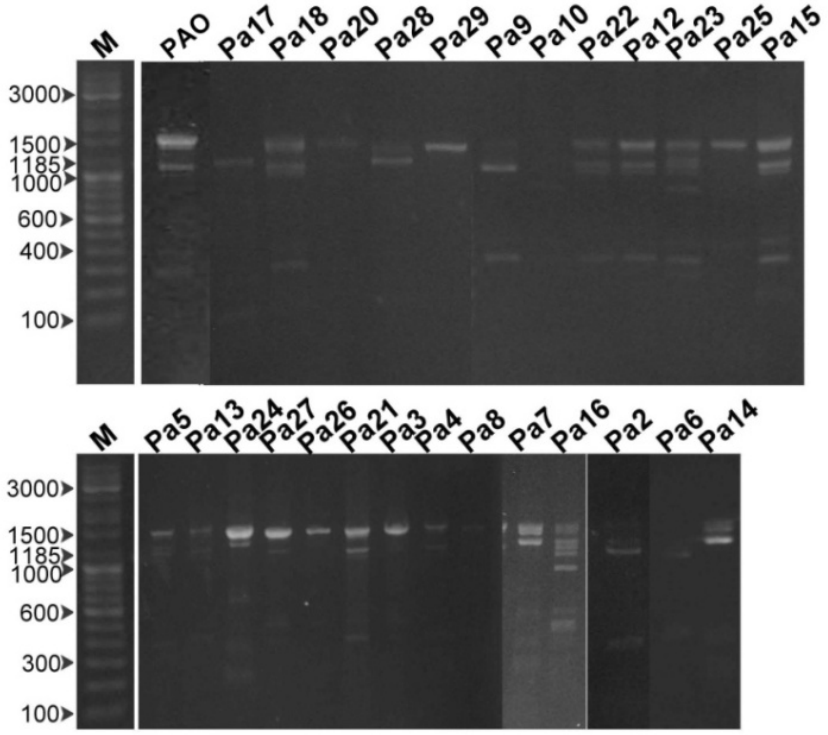


Fig. 4.31. Restriction profile of oprD amplicons from P. aeruginosa reference strain - MTCC-PAO and clinical isolates digested with HinC II.

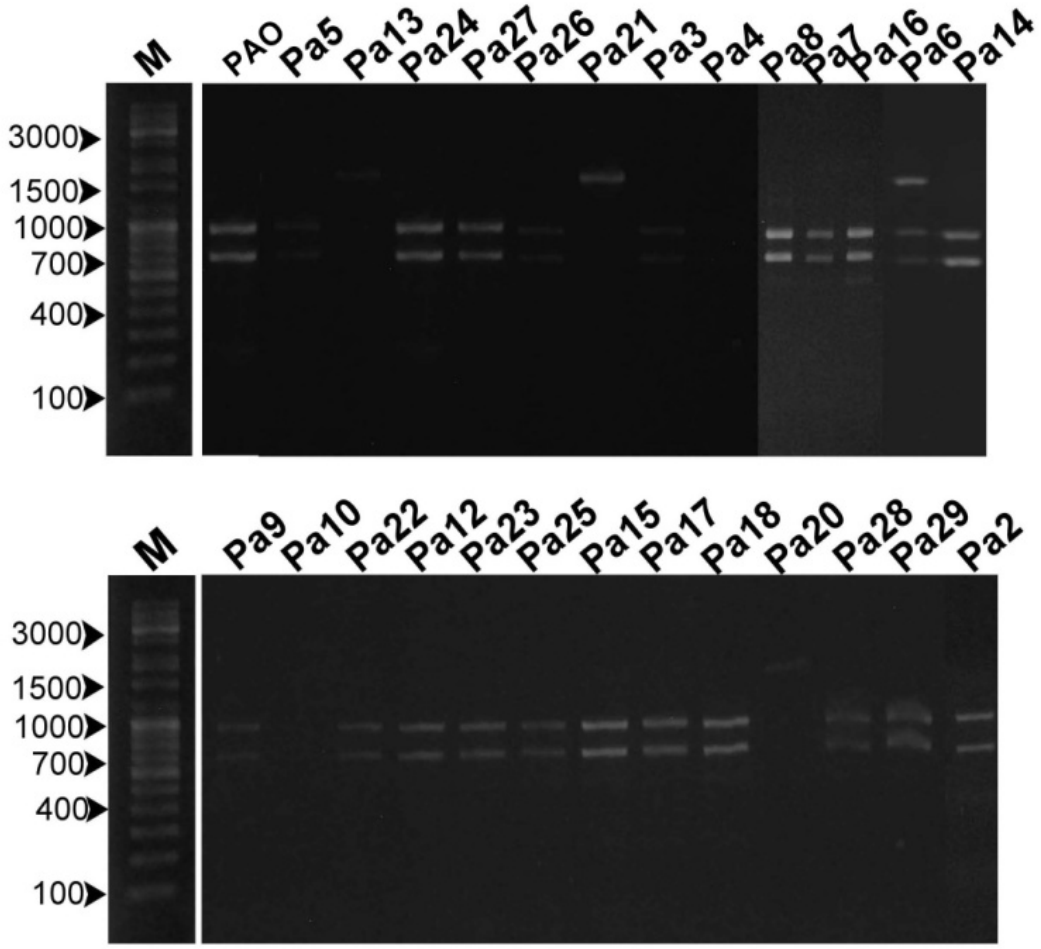


Fig. 4.32. Restriction profile of oprD amplicons from P. aeruginosa reference strain - MTCC-PAO and clinical isolates digested with Pst I.

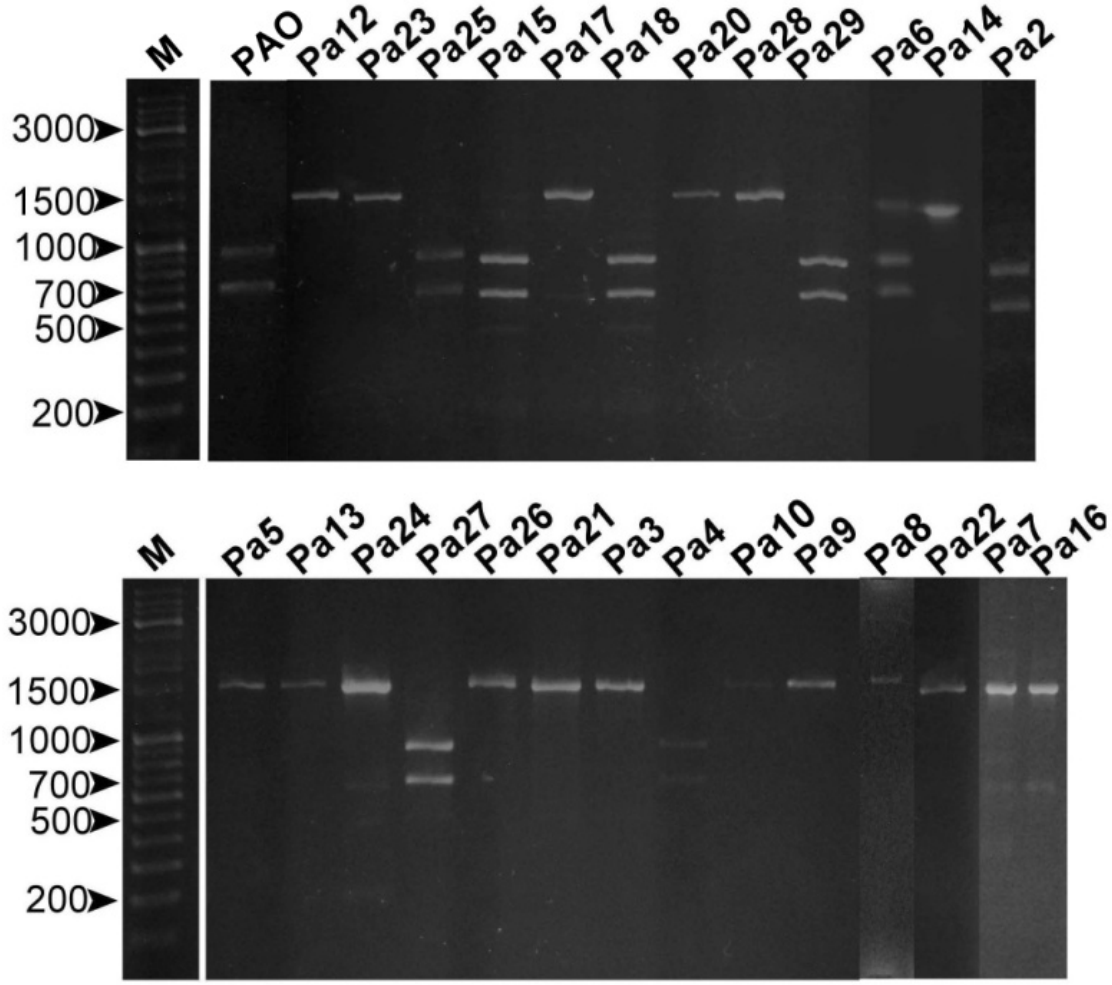


Fig. 4.33. Restriction profile of oprD amplicons from P. aeruginosa reference strain - MTCC-PAO and clinical isolates digested with Pvu II.

Table. 4.17 Details of restriction analysis of *oprD* amplicons from *P. aeruginosa* clinical isolates employing restriction endonucleases – *Hinc*II, *Pst* I and *Pvu* II

Isolates of OprD types	*HinC* II		*Pst* I		*Pvu* II	
	Bands (bp)	Specified mutations	Bands (bp)	Specified mutations	Bands (bp)	Specified mutations
A-Pa2	1500, 1185, >1000,400	-	1000, 700	-	1000,700	-
A-Pa6	1185, >1000,400	-	1500,1000 700	-	1500, 1000,700	-
A-Pa15	1500, 1185, >1000, 500,400	-	1000,700	-	1000,700, 500	-
A-Pa18	1500,1185,400	-	1000,700	-	1000,700,500	-
B1-Pa9	1500,1185,400	-	1000,700	-	1500	492#
B2-Pa29	1500	-	1000,700	-	1500,1000, 700	-
C1-Pa4	1500,1185,400	-	1000,700	-	1000,700	-
C2-Pa12	1500, 1185, >1000,400	-	1000,700	-	1500	492#
C2-Pa20	1500	-	1500	-	1500	492#
C2-Pa22	1500, 1185, >1000,400	-	1000,700	-	1500	492#
C3-Pa13	1500, 1185, >1000,400	-	1500	493#	1500	492#
C4-Pa21	1500, 1185, >1000, 500,400	-	1500	493#	1500	492#
D1-Pa16	1500, >1185, 1185, >1000,900 500,400	(ΔT at 1118)*	1000,700, 500	-	1500,500	492#
D2-Pa26	1500	192#, (ΔT at 1118)*	1000,700	-	1500	492#
D3-Pa7	1500,>1185	192#, (ΔT at 1118)*	1000,700	-	1500,500	492#
D3-Pa14	1500,>1185, 1185,400	192#, (ΔT at 1118)*	1000,700	-	1500	492#
D3-Pa17	1185	192#, (ΔT at 1118)*	1000,700	-	1500,700	492#
D3-Pa24	1500,>1185,700, 400,200	192#, (ΔT at 1118)*	1000,700	-	1500,700	492#
D3-Pa28	1500,>1185	192#, (ΔT at1118)*	1000,700	-	1500	492#
D4-Pa8	1500,1185	(ΔT at 1118)*	1000,700	-	1500	492#
D5-Pa5	1500,>1185,	(ΔT at	1000,700	-	1500	492#

	1185,400	1118)*				
D5-Pa23	1500, >1185, 1185, >1000,900 400,300	(ΔT at 1118)*	1000,700	-	1500	492#
D6-Pa3	1500, 1185,500	(ΔT at 1118)*	1000,700	-	1500	492#
E1-Pa25	1500,500	192#	1000,700	-	1000,700	
E2-Pa10	ND	(ΔT at 1118)*	ND	-	1500	492#
F-Pa27	1500,1185,500,400	-	1000,700	-	1000,700	-

*# Denotes Restriction Fragment Length Polymorphism (RFLP) generated at the specified positions due to mutations resulting in loss of restriction site; * denotes RFLP generated at the specified position due to mutations resulting in the creation of a restriction site; ND - restriction fragments were not detected in Pa10 due to extremely low, undetectable quantities of DNA; '-' represents no mutations.*

The loss and gain of restriction sites were clearly evident with respect to the three selected enzymes in the majority of the isolated mutants. In other words, the results demonstrated that the *oprD* amplicons from the clinical isolates exhibited Restriction Fragment Length Polymorphism (RFLP) and this could be used as a tool for detecting *P. aeruginosa* porin mutants. Since the oprD gene plays a key role in antibiotic resistance, the discovery of this RFLP in this study can be exploited to characterize clinical isolates without taking recourse to DNA sequencing.

CONCLUSIONS

The present study has enabled a better assessment and understanding of the status of drug resistance in clinical isolates of MDR gram-negative bacteria in the State of Kerala. Bacteria can attain antibiotic resistance through intrinsic or acquired mechanisms. Intrinsic resistance can be a result of impaired permeability of the bacterial envelope, efficient efflux pump systems, the absence or presence of low affinity antimicrobial target or the presence of enzymes which inhibit or destroy the antibiotics. Acquired resistance can either be a consequence of *de novo* mutations or may arise by horizontal gene transfer. A synergetic interplay between reduced uptake due to low permeability of outer membrane and active drug export through efflux pump activity limits the intracellular access of antibiotics in gram-negative bacteria which results in the development of MDR phenotype. Hence, the focus of this doctoral research was to gain deeper molecular insights into the regulation and expression of efflux pump and porin-related genes in clinical isolates of MDR *P. aeruginosa*. This study showcases the genotypic variations prevalent among the nosocomial pathogenic population of *P. aeruginosa* collected during the 2012-2016. The highlights of this research work have been summarized below:

- A total of 144 MDR gram-negative bacterial isolates collected from various clinical laboratories in Kerala were included in this study, which were found to represent four genera comprising of *Klebsiella* spp. (n=50), *E. coli* (n=46), *P. aeruginosa* (n=33) and *Acinetobacter* spp. (n=15).

- All 144 isolates were analysed for their resistance against pre-determined batteries of antibiotics. All strains were found to be ampicillin-resistant. Of these, *E. coli* strains also exhibited complete resistance to CTX, CAZ and CIP. Relatively low resistance was exhibited by *Klebsiella* spp. and *E. coli* against antibiotic C which was found to be 52% and 22% respectively. Notably, all *P. aeruginosa* isolates showed resistance against NA with only 48% of them being PB-resistant. All the 15 *Acinetobacter* spp. were also

found to display resistance against AT, CTX, CPM, CIP, PIT, TE and CL. The percentage of isolates resistant to all tested antibiotics, that is with a MAR index value of 1.0, was found to be 37% (n=17) of *Klebsiella* spp., 9% (n=4) of *E. coli,* 24% (n=8) of *P. aeruginosa* and 53% (n=8) of *Acinetobacter* spp.

- A preliminary screening of efflux pump-mediated drug-resistance using EtBr-agar cartwheel method revealed efflux activity in 21.5% (n=31, out of 144) of the isolates. Incidentally, on the basis of a thorough literature scan, this is the first report from the State of Kerala on efflux pump-mediated drug-resistance among MDR gram-negative bacteria. Of these, *P. aeruginosa* isolates showed much higher efflux pump activity in comparison to isolates from other genera. Among the 33 *P. aeruginosa* from a total of 144 isolates, as many as 17 effectively effluxed the fluorochrome dye (EtBr). Of the 31 isolates identified with efflux pump activity by the EtBr-agar cartwheel procedure, only 19 were confirmed to possess efflux pumps using an EPI-based microplate assay employing selected antibiotics against which resistance was observed. Of these, 7 isolates displayed efflux pump activity against more than one antibiotic. Further, efflux pump-mediated drug-resistance was found to be most prevalent in *P. aeruginosa* (27.3%, n=9), followed by that in *E. coli* (17.4%, n=8), *Acinetobacter* spp. (6.7%, n=1) and *Klebsiella* spp. (2%, n=1).

- All further investigations including phenotypic detection of various beta-lactamases, RAPD analysis, efflux pump and porin related gene expression/mutation studies were focused on 29* MDR *P. aeruginosa* designated as Pa1-Pa29 (*4 out of 33 isolates failed to revive)

- Beta-lactamases such as AmpC, ESBL and MBL positive *P. aeruginosa* isolates amounted to 31.0% (n=9), 13.8% (n=4) and 44.8% (n=13) respectively; 27.6% (n=8) were co-producers of AmpC and MBL with only a single isolate co-producing ESBL and MBL. About 41.4 % (n=12) of the

isolates were found lacking in the expression of any of the three enzymes tested.

- The dendrogram generated from RAPD profile of *P. aeruginosa* showed two major clusters - *A* and *B*, in which major cluster *A* was subdivided into *A1* and *A2* sub-clusters. Majority of the isolates expressing efflux pump activity phenotypically were found to be included in the sub-cluster *A1*. Isolates Pa15, Pa16, Pa20, Pa23, Pa24 and Pa25 possessing MAR index 1.0 fell under sub-cluster *A2*.

- Gene expression analysis of MDR *P. aeruginosa* in comparison to that of reference strain *P. aeruginosa* MTCC-PAO revealed that 10.3% (n=3), 20.7% (n= 6) and 10.3% (n=3) of isolates displayed increased transcription of efflux pump genes - mexB (~5-11 folds), *mexY* (~4- 26 folds) and *mexD* (~4-14 folds) respectively. Simultaneous expression of MexB and MexY was observed in Pa16 whilst Pa6 and Pa13 simultaneously expressed *mexB*, *mexY* and *mexD*. Expression of efflux pump gene - *mexF* in all of the 29 isolates was comparatively far lower than that obtained for the reference strain. The porin gene- *oprD* remained downregulated in 89.7% (n=26) of the isolates in comparison to the transcript levels of the reference strain.

- All isolates hyperexpressing Mex efflux pump systems were found to be devoid of ESBL production. AmpC and MBL production were observed in Mex efflux pump overproducing isolates Pa7, Pa13, Pa16, Pa25, and Pa29. Pa6 (coproducer of MexB, MexY and MexD) and Pa5 (MexY overproducer) were devoid of beta-lactamase enzymes such as AmpC and MBL. Strikingly, two isolates Pa1 and Pa2 in which efflux pump activity was confirmed by phenotypic EPI-based microplate assay tested negative for the presence of efflux pump related transcripts. This suggested that the efflux pumps other than the ones tested were operational. Alternatively, this could be due to the triggering of MexAB-OprM efflux pump under the experimental condition of antibiotic-induced oxidative stress. All the 26 porin down regulators were found to possess a MAR index of 1.0 or less.

Among them, seven isolates were found to show efflux pump activity, but only three, Pa7 (MexD overproducer), Pa16 (co-producer of both MexB and MexY) and Pa25 (MexY overproducer) showed a MAR index of 1.0. Of the porin down regulators, Pa20, Pa24 and Pa26 failed to express both beta-lactamase and efflux pump activity but still had a MAR index of 1.0. Notably, in Pa1, Pa11 and Pa19 where porin expression levels were found comparable to that of the reference strain, displayed a MAR index less than 1.0. As expected, these isolates are apparently equipped with other drug resistance mechanisms which needs further probing.

- MexAB overproducers Pa13 and Pa16 were found to harbour mutations in their repressor genes - *mexR*, *nalC* and *nalD*. Both transitional and transversional point mutations, either silent or leading to changes in amino acid substitutions were observed in *mexR* and *nalC* of Pa13 and Pa16 and in *nalD* of Pa16. The *nalD* of Pa13 displayed only silent mutations, whilst Pa6 was conspicuous by the absence of mutations of either type. The *mexR* of Pa16 isolate was found to harbour novel point mutations, ^{7}proline→leucine substitution, ^{143}proline→threonine substitution and a substitution of an *ochre* codon with that for serine. The gene also displayed a novel mutation involving insertion of a cysteine at the 444th base position, followed by an *opal* codon. The *nalD* of Pa16 was found to harbour a mutation at 153rd amino acid position (leucine→glutamine). Phylogenetic analysis of *mexR*, *nalC* and *nalD* concatenated sequence revealed that the sequence from Pa13 strain showed genetic similarity with those reported from Tamil Nadu (India) and Taiwan. Interestingly, the concatenated sequence of Pa16 isolate showed similarity with the sequence of *P.aeruginosa* first reported with *bla*NDM-1 in North America (GenBank accession no. CP012901.1) isolated from an elderly Canadian patient who was directly transferred to Calgary hospital, Alberta, Canada, following a prolonged hospital stay in New Delhi, India. High mobility of people thus provides ample scope for widespread dissemination of drug-resistant microbes with extensive phylogenetic divergence.

- Sequences obtained from the *mexZ* gene amplicons of MexXY overproducers showed a variety of silent/point mutations. Point mutations in *mexZ* gene of two isolates, Pa16 (^{2}alanine→threonine) and Pa29 (^{52}tyrosine → cysteine) which might be responsible for upregulation of MexXY.

- DNA sequences obtained from *nfxB* gene amplicons of three MexCD-OprJ overproducers Pa6, Pa7 and Pa13 showed various synonymous and non-synonymous mutations. Mutation analysis revealed ^{80}leucine → serine substitution in Pa6, ^{117}serine→proline substitution in Pa7 and ^{5}serine→ phenylalanine substitution in Pa13. Notably, mutations in Pa6 and Pa7 isolates have been found to be novel and have not yet been reported in GenBank data base. Phylogenetic analysis revealed that the sequence from Pa6 showed similarity with isolates from Taiwan, Mexico and Brazil. Pa13 isolate displayed similarity with that reported from Tamil Nadu whilst Pa7 was found to be similar to *P.aeruginosa* sequences deposited from North America.

- Occurrence of an 8-bp deletion (GGCCAGCC) at nucleotide position 235 within a 14-bp direct repeat was found in all 24 out of 26 porin down regulators except in Pa2 and Pa6 and the reference strain MTCC- PAO. Phylogram constructed using 24 isolates tested in this study along with those retrieved from public data base showed genetic relatedness. Pa10, Pa16 and Pa23 were found to be similar to those isolates reported from North America. Pa12, Pa20 and Pa22 exhibited similarity with those from Taiwan and Pa13 and Pa21 were related to isolates from Tamil Nadu.

- On the basis of mutational patterns of the *oprD* gene, all 26 isolates displaying porin down regulation were classified into several types such as A, B1- 2, C1-4, D1-6, E1-2 and F. Isolates - Pa2, Pa6, Pa15 and Pa18 grouped under OprD type-A were found to be identical with the reference strain PAO1. Types B1 and B2 bore full length amino acid sequences except a change due to single amino acid substitution. C1 - 4 OprD types

showed full length OprD amino acid sequences with several polymorphisms at the amino acid level. Isolates of D1-6, OprD types showed a wide range of aminoacid substitutions along with nucleotide deletions and amino acid changes at 372 - 383 positions [372nd (VDSSSS-YAGL-) to 383rd]. E1-2 types displayed several amino acid changes but were conspicuous due to the presence of premature stop codons which resulted in truncated porin proteins. Pa25 isolate, representative of E1 type, carried a point mutation at the 708th nucleotide (T→G) position leading to formation of a premature stop codon, whilst premature termination was found to occur in Pa10 of E2 type by a point mutation at 1016th nucleotide position (G→A). The only isolate designated as F-type (Pa27) also displayed a premature stop codon.

- Phylogenetic analysis of *oprD* genes from *P. aeruginosa* isolates studied revealed similarities with those reported from France, Brazil, Taiwan, Singapore and North America. Interestingly, the *oprD* mutant Pa25 producing truncated porin protein was epidemiologically related to a strain reported from Malaysia.

- 3D models of the four selected OprD porin protein mutants Pa12, Pa7, Pa25 and Pa27 along with that of the control strain PAO1were built by using homology modelling approach, and validated using bioinformatics tools. The molecular modelling of the truncated porin proteins from Pa25 and Pa27 are visually dramatic due to the loss of substantial portions of the polypeptide.

- The *oprD* amplicons from majority of the clinical isolates were found to exhibit restriction fragment length polymorphism (RFLP) with respect to the three selected enzymes *Hinc* II, *Pst* I and *Pvu* II. Since the oprD gene plays a key role in antibiotic resistance, this RFLP discovered in this study has future potential to be used as a diagnostic tool for detection of *P. aeruginosa* porin mutants in clinical laboratories.

FUTURE PROSPECTIVES

An estimated 10% of the 30 million Kerala populations live overseas resulting in high mobility of people breaching geographical boundaries. Kerala State is also a well known tourist destination including medical tourism. High mobility of people thus provides ample scope for widespread dissemination of drug-resistant microbes.

The present study has been successful in unraveling vital information on the molecular biology of multidrug resistance in clinically relevant gram-negative bacteria, especially with respect to MDR *P. aeruginosa* isolates from within the State of Kerala. This study happens to be the first of its kind to provide molecular evidence and an insightful understanding of a tripartite protein nanomachine – the efflux pump which actively pumps out the antibiotics even before they start their destructive activity. The genes encoding the protein components of the efflux pumps and the regulatory genetic circuitry have been studied in detail. Of the various routes of drug entry into bacteria, membrane-bound protein channels such as porins play a pivotal role in facilitating influx of antibiotics. This study also sheds light on molecular mechanisms critical to porin gene expression and its regulation.

Given the fact that the multidrug resistant clinical isolates were collected from various clinical laboratories in Kerala State, this investigation clearly provides a window view of the present scenario of drug resistance linked with efflux pump mechanism and its relation to porin downregulation. From this perspective, the current situation clearly warrants the urgent need for advanced molecular studies to determine the contributions of other resistance mechanisms as well. For instance this study clearly showed that the overall percentage of multidrug resistance was least represented in the genera of *Acinetobacter* spp., but they were found to display potent antibiotic resistance. This necessitates further expansion in terms of larger sampling followed by a critical analysis of multiple relevant loci in clinical isolates of *Acinetobacter* spp. including other nosocomial pathogens. Further, docking studies in efflux pump proteins may pave the way for the discovery of bacterial

resistance modifiers such as efflux pump inhibitors. Detailed studies on efflux pump inhibitors derived from natural bioresources including plants are an attractive option. Their potential to work in synergy with the existing regimes of antibiotics may open new avenues for extensions of the present study. Such approaches may thereby facilitate re-introduction of therapeutically ineffective antimicrobial agents into clinical use and may prove beneficial to effectively control and check emergence of multi-drug resistance.

REFERENCE

Ahmed SM, Jakribettu RP, Kottakutty S, Arya B, Shakir VPA. (2012) An emerging multidrug resistant pathogen in a tertiary care centre in North Kerala. Annals of Biological Research 3, 2794-2799.

Alekshun MN, Levy SB. (2007) Molecular mechanisms of antibacterial multidrug resistance. Cell 128, 1037-1050.

Alguel Y, Lu D, Quade N, Sauter S, Zhang X. (2010) Crystal structure of MexZ, a key repressor responsible for antibiotic resistance in *Pseudomonas aeruginosa*. Journal of Structural Biology 172, 305–310.

Alibert S, N′Gompaza Diarra J, Hernandez J, Stutzmann A, Fouad M, Boyer G, Pages JM. (2017) Multidrug efflux pumps and their role in antibiotic and antiseptic resistance: a pharmacodynamic perspective. Expert Opinion on Drug Metabolism and Toxicology 13, 301–309.

Allcock S, Young EH, Holmes M, Gurdasani D, Dougan G, Sandhu MS, Solomon L, Török ME. (2017) Antimicrobial resistance in human populations: challenges and opportunities. Global Health, Epidemiology and Genomics 2, e4.

Alvarez-Ortega C, Olivares J, Martínez JL. (2013) RND multidrug efflux pumps: what are they good for?. Fronietiers in Microbiology 4, 7.

Ambler RP. (1980) The structure of beta-lactamases. Philosophical Transactions of the Royal Society B 289, 321–331.

Aminov RI. (2010) A brief history of the antibiotic era: lessons learned and challenges for the future. Frontiers in microbiology 1.

Ansari S, Dhital R, Shrestha S, Thapa S, Puri R, Chaudhary N, Khatiwada S, Gautam R. (2016) Growing menace of antibacterial resistance in clinical

isolates of *Pseudomonas aeruginosa* in Nepal: an insight of beta-lactamase production. BioMed Research International 2016,8.

Askoura M, Mottawea W, Abujamel T, Taher I. (2011) Efflux pump inhibitors (EPIs) as new antimicrobial agents against *Pseudomonas aeruginosa*. The Libyan Journal of Medicine 6, 10.3402/ljm.v6i0.5870.

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K. (1995) Short Protocols in Molecular Biology. 3 rdedn. John Wiley & Sons Inc. pp 2.11- 2.12.

Balasubramanian D, Schneper L, Merighi M, Smith R, Narasimhan G, Lory S, Mathee K. (2012) The regulatory repertoire of *Pseudomonas aeruginosa* AmpC beta-lactamase regulator AmpR includes virulence genes. PLoS One 7, e34067.

Baron EJ, Peterson LR, Finegold SM. (1994) Bailey and Scott's Diagnostic Microbiology. 9th edn. St. Louis: The C V Mosby Company.

Benkert P, Künzli M, Schwede T. (2009) QMEAN server for protein model quality estimation. Nucleic Acids Research 37(Web Server issue),W510–W514.

Biswas S, Mohammad MM, Patel DR, Movileanu L, van den Berg B. (2007) Structural insight into OprD substrate specificity. Nature Structural & Molecular Biology 14, 1108-1109.

Black JA, Moland ES, Thomson KS. (2005) AmpC disc test for detection of plasmid-mediated AmpC beta-lactamases in *Enterobacteriaceae* lacking chromosomal AmpC beta-lactamases. Journal of Clinical Microbiology 43, 3110-3113.

Blair JM, Piddock LJ. (2009) Structure, function and inhibition of RND efflux pumps in gram-negative bacteria: an update. Current Opinion in Microbiology 12, 512–519.

Blair JM, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJ. (2015) Molecular mechanisms of antibiotic resistance. Nature Reviews Microbiology 13, 42–51.

Bosch F, Laia R. (2008) The contributions of Paul Ehrlich to pharmacology: a tribute on the occasion of the centenary of his Nobel prize. Pharmacology 82, 171–179.

Braz VS, Furlan JP, Fernandes AF, Stehling EG. (2016) Mutations in NalC induce MexAB-OprM overexpression resulting in high level of Aztreonam resistance in environmental isolates of *Pseudomonas aeruginosa*. FEMS Microbiology Letters 363.

Bush K, Jacoby GA. (2010) Updated functional classification of beta-lactamases. Antimicrobial Agents and Chemotherapy 54, 969-976.

Cabot G, Zamorano L, Moyà B, Juan C, Navas A, Blázquez J, Oliver A. (2016) Evolution of *Pseudomonas aeruginosa* antimicrobial resistance and fitness under low and high mutation rates. Antimicrobial Agents Chemotherapy 60, 1767–1778.

Cai B, Echols R, Magee G, Ferreira JCA, Morgan G, Ariyasu M, Sawada T, Nagata TD. (2017) Prevalence of carbapenem-resistant gram-negative infections in the United States predominated by *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. Open Forum Infectious Diseases 4, ofx176.

Cao L, Srikumar R, Poole K. (2004) MexAB-OprM hyperexpression in NalC-type multidrug-resistant *Pseudomonas aeruginosa*: identification and characterization of the nalC gene encoding a repressor of PA3720-PA3719. Molecular Microbiology 53, 1423–1436.

Castang S, McManus HR, Turner KH, Dove SL. (2008) H-NS family members function coordinately in an opportunistic pathogen. Proceedings of the National Academy of Sciences of the United States of America 105, 18947–18952.

CDDEP. (2017) Scoping report on antimicrobial resistance in India. Washington, DC: Center for Disease Dynamics, Economics & Policy.

Chatterjee M, Anju C, Biswas L, Kumar VA, Mohan CG, Biswas R. (2016) Antibiotic resistance in *Pseudomonas aeruginosa* and alternative therapeutic options. International Journal of Medical Microbiology 306, 48–58.

Chaudhary AS. (2016) A review of global initiatives to fight antibiotic resistance and recent antibiotics discovery. Acta Pharmaceutica Sinica B 6, 552–556.

Chellat MF, Raguz L, Riedl R. (2016) Targeting antibiotic resistance. Angewandte Chemie International Edition in English 55, 6600–6626.

Chen H, Hu J, Chen PR, Lan L, Li Z, Hicks LM, Dinner AR, He C. (2008) The *Pseudomonas aeruginosa* multidrug efflux regulator MexR uses an oxidation-sensing mechanism. Proceedings of the National Academy of Sciences of the United States of America 105, 13586–13591.

Chen W, Wang D, Zhou W, Sang H, Liu X, Ge Z, Zhang J, Lan L, Yang C-G, Chen H. (2016) Novobiocin binding to NalD induces the expression of the MexAB-OprM pump in *Pseudomonas aeruginosa*. Molecular Microbiology 100, 749–758.

Choudhury D, Ghose A, Chanda DD, Talukdar AD, Choudhury MD, Paul D, Maurya A P, Chakravarty A, Bhattacharjee A. (2016) Correction: Premature termination of MexR leads to overexpression of MexAB-OprM efflux pump in *Pseudomonas aeruginosa* in a tertiary referral hospital in India. PLoS ONE 11, e0151308.

Choudhury R, Panda S, Singh DV. (2012) Emergence and dissemination of antibiotic resistance: a global problem. Indian Journal of Medical Microbiology 30, 384–390.

Christen M, Hünenberger PH, Bakowies D, Baron R, Bürgi R, Geerke DP, Heinz TN, Kastenholz MA, Kräutler V, Oostenbrink C, Peter C, Trzesniak D, van Gunsteren WF. (2005) The GROMOS Software for Biomolecular

Simulation: GROMOS05. Journal of Computational Chemistry 26, 1719–1751.

CLSI. (2012) Performance standards for antimicrobial susceptibility testing: twenty-second informational supplement. National Committee for Clinical Laboratory Standards.

Couce A, Rodríguez-Rojas A, Blázquez J. (2016) Determinants of genetic diversity of spontaneous drug resistance in bacteria. Genetics 203, 1369–1380.

Das B, Chaudhuri S, Srivastava R, Nair GB, Ramamurthy T. (2017) Fostering research into antimicrobial resistance in India. BMJ 358, j3535.

Daury L, Orange F, Taveau JC, Verchere A, Monlezun L, Gounou C, Marredy RKR, Picard M, Broutin I, Pos KM, Lambert O. (2016) Tripartite assembly of RND multidrug efflux pumps. Nature Communications 7, 10731.

Davies J, Davies D. (2010) Origins and evolution of antibiotic resistance. Microbiology and Molecular Biology Reviews 74, 417-433.

Debabov D. (2013) Antibiotic resistance: origins, mechanisms, approaches to counter. Applied Biochemistry and Microbiology 49, 665-671.

Delcour AH. (2009) Outer membrane permeability and antibiotic resistance. Biochimica et Biophysica Acta 1794, 808–816.

Dreier J, Ruggerone P. (2015) Interaction of antibacterial compounds with RND efflux pumps in *Pseudomonas aeruginosa.* Frontiers in Microbiology 6, 660.

Dridi B, Lupien A, Bergeron MG, Leprohon P, Ouellette M. (2015) Differences in antibiotic-induced oxidative stress responses between laboratory and clinical isolates of *Streptococcus pneumoniae*. Antimicrobial Agents Chemotherapy 59, 5420–5426.

Du D, van Veen HW, Murakami S, Pos KM, Luisi BF. (2015) Structure, mechanism and cooperation of bacterial multidrug transporters. Current Opinion in Structural Biology 33, 76–91.

Dumas JL, van Delden C, Perron K, Köhler T. (2006) Analysis of antibiotic resistance gene expression in *Pseudomonas aeruginosa* by quantitative real-time-PCR. FEMS Microbiology Letters 254, 217–225.

Džidić S, Šušković J, Kos B. (2008) Antibiotic resistance mechanisms in bacteria: biochemical and genetic aspects. Food Technology and Biotechnology 46, 11-21.

Easwaran S, Yerat RC, Ramaswamy R. (2016) A study on detection of extended-spectrum beta-lactamases (ESBLs) and comparison of various phenotypic methods of AmpC detection in *Pseudomonas aeruginosa* from various clinical isolates in a tertiary care teaching hospital. Muller Journal of Medical Sciences and Research 7, 35-39.

Edalucci E, Spinelli R, Dolzani L, Riccio ML, Dubois V, Tonin EA, Rossolini GM, Lagatolla C. (2008) Acquisition of different carbapenem resistance mechanisms by an epidemic clonal lineage of *Pseudomonas aeruginosa*. Clinical Microbiology and Infection 14, 88–90.

Ellappan K, Narasimha HB, Kumar S. (2018) Coexistence of multidrug resistance mechanisms and virulence genes in carbapenem-resistant *Pseudomonas aeruginosa* strains from a tertiary care hospital in South India. Journal of Global Antimicrobial Resistance 12 , 37-43.

Epp SF, Köhler T, Plésiat P, Michéa-Hamzehpour M, Frey J, Pechère J-C. (2001) C-terminal region of *Pseudomonas aeruginosa* outer membrane porin OprD modulates susceptibility to meropenem. Antimicrobial Agents and Chemotherapy 45, 1780–1787.

Fang ZL, Zhang LY, Huang YM, Qing Y, Cao KY, Tian GB, Huang X. (2014) OprD mutations and inactivation in imipenem-resistant *Pseudomonas*

aeruginosa isolates from China. Infection, Genetics and Evolution 21, 124–128.

Farooqui H, Jit M, Heymann DL, Zodpey S. (2015) Burden of severe pneumonia, pneumococcal pneumonia and pneumonia deaths in Indian states: modeling based estimates. PLoS One 10, e0129191.

Fernandes R, Amador P, Prudêncio C. (2013) Beta-lactams: chemical structure, mode of action and mechanisms of resistance. Reviews in Medical Microbiology 24, 7–17.

Fernández L, Hancock REW. (2012) Adaptive and mutational resistance: role of porins and efflux pumps in drug resistance. Clinical Microbiology Reviews 25, 661–681.

Fernando DM, Kumar A. (2013) Resistance-nodulation-division multidrug efflux pumps in gram-negative bacteria: role in virulence. Antibiotics 2, 163-181.

Fraud S, Campigotto AJ, Chen Z, Poole K. (2008) MexCD-OprJ multidrug efflux system of *Pseudomonas aeruginosa*: involvement in chlorhexidine resistance and induction by membrane-damaging agents dependent upon the AlgU stress response sigma factor. Antimicrobial Agents and Chemotherapy 52, 4478-4482.

Galán J-C, González-Candelas F, Rolain J-M, Cantón R. (2013) Antibiotics as selectors and accelerators of diversity in the mechanisms of resistance: from the resistome to genetic plasticity in the beta-lactamases world. Frontiers in Microbiology 4, 9.

Galdiero S, Falanga A, Cantisani M, Tarallo R, Pepa MED, D'Oriano V, Galdiero M. (2012) Microbe-host interactions: structure and role of gram-negative bacterial porins. Current Protein & Peptide Science 13, 843–854.

Gelband H, Miller-Petrie M, Pant S, Gandra S, Levinson J, Barter D, White A, Laxminarayanan R. (2015) The State of the World's Antibiotics. Washington, DC: Centre for Disease Dynamics, Economics & Policy.

Giedraitienė A, Vitkauskienė A, Naginienė R, Pavilonis A. (2011) Antibiotic resistance mechanisms of clinically important bacteria. Medicina (Kaunas) 47, 137–146.

Gniadek TJ, Carroll KC, Simner PJ. (2016) Carbapenem-resistant non-glucose-fermenting gram-negative bacilli: the missing piece to the puzzle. Journal of Clinical Microbiology 54, 1700-1710.

Gould K. (2016) Antibiotics: from prehistory to the present day. Journal of Antimicrobial Chemotherapy 71, 572–575.

Grosso-Becerra M-V, Santos-Medellín C, González-Valdez A, Méndez J-L, Delgado G, Morales-Espinosa R, Servín-González L, Alcaraz L-D, Soberón-Chávez G. (2014) *Pseudomonas aeruginosa* clinical and environmental isolates constitute a single population with high phenotypic diversity. BMC Genomics 15, 318.

Hammami S, Ghozzi R, Burghoffer B, Arlet G, Redjeb S. (2009) Mechanisms of carbapenem resistance in non-metallo-beta-lactamase-producing clinical isolates of *Pseudomonas aeruginosa* from a Tunisian hospital. Pathologie Biologie (Paris) 57, 530–535.

Hassan KA, Liu Q, Henderson PJ, Paulsen IT. (2015) Homologs of the *Acinetobacter baumannii* AceI transporter represent a new family of bacterial multidrug efflux systems. MBio 6, e01982–14.

Hassan MT, van der Lelie D, Springael D, Romling U, Ahmed N, Mergeay M. (1999) Identification of a gene cluster, *czr*, involved in cadmium and zinc resistance in *Pseudomonas aeruginosa*. Gene 238, 417– 425.

Hora S, Ali Z. (2012) A study of relationship between antibiotic resistance and molecular characteristics of *Escherichia coli* isolates obtained from different human clinical specimens against multiple antibiotic resistance (MAR) Index in Bareilly (India) Region. International Journal of Pharmaceutical Sciences and Research 3, 3331-3336.

Huang H, Hancock RE. (1996) The role of specific surface loop regions in determining the function of the imipenem-specific pore protein OprD of *Pseudomonas aeruginosa*. Journal of Bacteriology 178, 3085–3090.

Huelsenbeck JP, Ronquist F. (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754–755.

IIMAR (Indian initiative for management of antibiotic resistance). (2016) http://save-antibiotics.blogspot.com/.

Islam S, Jalal S, Wretlind B. (2004) Expression of the MexXY efflux pump in amikacin- resistant isolates of *Pseudomonas aeruginosa*. Clinical Microbiology and Infection 10, 877-883.

Jahandideh S. (2013) Diversity in structural consequences of MexZ mutations in *Pseudomonas aeruginosa*. Chemical Biology and Drug Design 81, 600–606.

Jochumsen N, Marvig RL, Damkiær S, Jensen RL, Paulander W, Molin S, Jelsbak L, Folkesson A. (2016) The evolution of antimicrobial peptide resistance in *Pseudomonas aeruginosa* is shaped by strong epistatic interactions. Nature Communications 7, 13002.

Kabsch W, Sander C. (1983) Dictionary of protein secondary structure: Pattern recognition of hydrogen-bonded and geometrical features. Biopolymers 22, 2577–2637.

Kao C-Y, Chen S-S, Hung K-H, Wu H-M, Hsueh P-R, Yan J-J, Wu J-J. (2016) Overproduction of active efflux pump and variations of OprD dominate in imipenem-resistant *Pseudomonas aeruginosa* isolated from patients with bloodstream infections in Taiwan. BMC Microbiology, 16, 107.

Kim CH, Kang HY, Kim BR, Jeon H, Lee YC, Lee SH, Lee JC. (2016) Mutational inactivation of OprD in carbapenem-resistant *Pseudomonas aeruginosa* isolates from Korean hospitals. Journal of Microbiology 54, 44–49.

Köhler T, Epp SF, Curty LK, Peche`re JC. (1999) Characterization of MexT, the regulator of the MexE-MexF-OprN multidrug efflux system of *Pseudomonas aeruginosa*. Journal of Bacteriology 181, 6300–6305.

Köhler T, Michea-Hamzehpour M, Henze U, Gotoh N, Curty LK, Pechere JC. (1997) Characterization of MexE-MexFOprN, a positively regulated multidrug efflux system of *Pseudomonas aeruginosa.* Molecular Microbiology. 23, 345-354.

Kos VN, McLaughlin RE, Gardner HA. (2016) Identification of unique in-frame deletions in OprD among clinical isolates of *Pseudomonas aeruginosa*. Pathogens and Disease 74, ftw031.

Krishna MP, Varghese R, Thomas DM, Hatha AAM. (2014) Multiple antibiotic resistance of environmental bacteria isolated from heavy metal polluted industrial region. Trakia Journal of Sciences 12, 109-113.

Krumperman PH. (1983) Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. Applied and Environmental Microbiology 46,165–170.

Kumar A, Schweizer HP. (2005) Bacterial resistance to antibiotics: active efflux and reduced uptake. Advanced Drug Delivery Reviews 57, 1486–1513.

Kumar S, Floyd JT, He G, Varela MF. (2013) Bacterial antimicrobial efflux pumps of the MFS and MATE transporter families: A review. Recent Research Developments in Antimicrobial Agents & Chemotherapy 7, 1-21.

Kumar SG, Adithan C, Harish BN, Sujatha S, Roy G, Malini A. (2013) Antimicrobial resistance in India: A review. Journal of Natural Science, Biology and Medicine 4, 286-291.

Laskowski RA, Macarthur MW, Thornton JM. (1993) PROCHECK:a program to check the stereochemical quality of protein structures. Journal of Applied Crystallography 26, 283-291.

Laudy AE, Róg P, Smolińska-Król K, Ćmiel M, Słoczyńska A, Patzer J, Dzierżanowska D, Wolinowska R, Starościak B, Tyski S. (2017) Prevalence of ESBL-producing *Pseudomonas aeruginosa* isolates in Warsaw, Poland, detected by various phenotypic and genotypic methods. PLoS ONE 12, e0180121.

Laxminarayan R, Brown GM. (2001) Economics of antibiotic resistance: A theory of optimal use. Journal of Environmental Economics and Management 42, 183–206.

Laxminarayan R, Chaudhury RR. (2016) Antibiotic resistance in India: Drivers and opportunities for action. PLoS Medicine 13, e1001974.

Lee JY, Ko KS. (2012) OprD mutations and inactivation, expression of efflux pumps and AmpC, and metallo-β-lactamases in carbapenem-resistant *Pseudomonas aeruginosa* isolates from South Korea. International Journal of Antimicrobial Agents 40, 168–172.

Lesch JE. (2007) The first miracle drugs : how the sulfa drugs transformed medicine. Oxford: Oxford University Press.

Lewis K. (2013) Platforms for antibiotic discovery. Nature reviews Drug discovery 12, 371-387.

Lewis VG, Ween MP, McDevitt CA. (2012) The role of ATP-binding cassette transporters in bacterial pathogenicity. Protoplasma 249, 919–942.

Li H, Luo Y-F, Williams BJ, Blackwell TS, Xie C-M. (2012) Structure and function of OprD protein in *Pseudomonas aeruginosa*: from antibiotic resistance to novel therapies. International Journal of Medical Microbiology 302, 10.1016/j.ijmm.2011.10.001.

Li X-Z, Plésiat P, Nikaido H. (2015) The Challenge of efflux-mediated antibiotic resistance in gram-negative bacteria. Clinical Microbiology Reviews 28, 337–418.

Liao J, Schurr MJ, Sauer K. (2013) The MerR-like regulator BrlR confers biofilm tolerance by activating multidrug efflux pumps in *Pseudomonas aeruginosa* biofilms. Journal of Bacteriology 195, 3352-3363.

Lim D, Poole K, Strynadka NC. (2002) Crystal structure of the MexR repressor of the *mexRAB-oprM* multidrug efflux operon of *Pseudomonas aeruginosa*. Journal of Biological Chemistry 277**,** 29253-29259.

Lister PD, Wolter DJ, Hanson ND. (2009) Antibacterial- resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. Clinical Microbiology Reviews 22, 582–610.

Livermore DM. (2002) Multiple mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa*: our worst nightmare?. Clinical Infectious Diseases 34, 634–640.

Liwa AC, Jaka H. (2015) Antimicrobial resistance: Mechanisms of action of antimicrobial agents. The Battle Against Microbial Pathogens: Basic Science, Technological Advances and Educational Programs 2, 876-885.

Llanes C, Hocquet D, Vogne C, Benali-Baitich D, Neuwirth C, Plésiat P. (2004) Clinical strains of *Pseudomonas aeruginosa* overproducing MexAB-OprM and MexXY efflux pumps simultaneously. Antimicrobial Agents and Chemotherapy 48, 1797–1802.

Logan LK, Weinstein RA. (2017) The epidemiology of carbapenem-resistant *Enterobacteriaceae*: the impact and evolution of a global menace. Journal of Infectious Diseases 215, S28-S36.

Lomovskaya O, Watkins W. (2001) Inhibition of efflux pumps as a novel approach to combat drug resistance in bacteria. Journal of Molecular Microbiology and Biotechnology 3, 225–236.

López-Causapé C, Sommer LM, Cabot G, Rubio R, Ocampo-Sosa AA, Johansen HK, Figuerola J, Cantón R, Kidd TJ, Molin S, Oliver A. (2017) Evolution of

the *Pseudomonas aeruginosa* mutational resistome in an international cystic fibrosis clone. Scientific Reports 7, 5555.

Manjusha S, Sarita GB. (2011) Plasmid associated antibiotic resistance in *Vibrios* isolated from coastal waters of Kerala. International Food Research Journal 18, 1171-1181.

Manohar P. Shanthini T, Ayyanar R Bozdogan B, Wilson A, Tamhankar AJ, Nachimuthu R, Lopes BS. (2017) The distribution of carbapenem-and colistin-resistance in gram-negative bacteria from the Tamil Nadu region in India. Journal of Medical Microbiology 66, 874-883.

Marshall BM, Levy SB. (2011) Food Animals and Antimicrobials: Impacts on Human Health. Clinical Microbiology Reviews 24, 718–733.

Martins M, Couto I, Viveiros M, Amaral L. (2010) Identification of efflux-mediated multi-drug resistance in bacterial clinical isolates by two simple methods. Methods in Molecular Biology 642, 143-57.

Martins M, Viveiros M, Couto I, Costa SS, Pacheco T, Fanning S. (2011) Identification of efflux pump-mediated multidrug-resistant bacteria by the Ethidium bromide-agar Cartwheel method. In vivo 25, 171–178.

Maseda H, Saito K, Nakajima A, Nakae T. (2000) Variation of the mexT gene, a regulator of the MexEF-oprN efflux pump expression in wild-type strains of *Pseudomonas aeruginosa*. FEMS Microbiology Letters 192, 107–112.

Maseda H, Sawada I, Saito K, Uchiyama H, Nakae T, Nomura N. (2004) Enhancement of the *mexAB-oprM* efflux pump expression by a quorum-sensing autoinducer and its cancellation by a regulator, MexT, of the *mexEF-oprN* efflux pump operon in *Pseudomonas aeruginosa*. Antimicrobial Agents and Chemotherapy 48, 1320–1328.

Masi M, Réfrégiers M, Pos KM, Pagès JM. (2017) Mechanisms of envelope permeability and antibiotic influx and efflux in gram-negative bacteria. Nature Microbiology 2, 17001.

Masuda N, Sakagawa E, Ohya S, Gotoh N, Tsujimoto H, Nishino T. (2000) Contribution of the MexX-MexY-OprM efflux system to intrinsic resistance in *Pseudomonas aeruginosa*. Antimicrobial Agents and Chemotherapy 44, 2242–2246.

Mataseje LF, Peirano G, Church DL, Conly J, Mulvey M, Pitout JD. (2016) Colistin-nonsusceptible *Pseudomonas aeruginosa* sequence type 654 with $bla_{\text{NDM-1}}$ arrives in North America. Antimicrobial Agents and Chemotherapy 60, 1794–1800.

Melo F, Devos D, Depiereux E, Feytmans E. (1997) ANOLEA: A www server to assess protein structures. Proceedings of the 8th International Conference on Intelligent Systems for Molecular Biology 5, 187–190.

Miller SI. (2016) Antibiotic resistance and regulation of the gram-negative bacterial outer membrane barrier by host innate immune molecules. mBio 7, e01541-16.

Mima T, Kohira N, Li Y, Sekiya H, Ogawa W, Kuroda T, Tsuchiya T. (2009) Gene cloning and characteristics of the RND-type multidrug efflux pump MuxABC-OpmB possessing two RND components in *Pseudomonas aeruginosa*. Microbiology 155, 3509–3517.

Minagawa S, Inami H, Kato T, Sawada S, Yasuki T, Miyairi S, Horikawa M, Okuda J, Gotoh N. (2012) RND type efflux pump system MexAB-OprM of *Pseudomonas aeruginosa* selects bacterial languages, 3-oxo-acyl-homoserine lactones, for cell-to-cell communication. BMC Microbiology 12, 70.

Molton JS, Tambyah PA, Ang BS, Ling ML, Fisher DA. (2013) The global spread of healthcare-associated multidrug-resistant bacteria: A perspective from Asia. Clinical Infectious Diseases 56, 1310–1318.

Moore JD, Gerdt JP, Eibergen NR, Blackwell HE. (2014) Active efflux influences the potency of quorum sensing inhibitors in *Pseudomonas aeruginosa*. Chembiochem 15, 435–442.

Moreira MAS, Souza ECD, Moraes CAD. (2004) Multidrug efflux systems in gram-negative bacteria. Brazilian Journal of Microbiology 35, 19–28.

Morita Y, Cao L, Gould VC, Avison MB, Poole K. (2006) *nalD* encodes a second repressor of the mexAB-oprM multidrug efflux operon of *Pseudomonas aeruginosa*. Journal of Bacteriology 188, 8649–8654.

Morita Y, Murata T, Mima T, Shiota S, Kuroda T, Mizushima T, Gotoh N, Nishino T, Tsuchiya T. (2003) Induction of *mexCD-oprJ* operon for a multidrug efflux pump by disinfectants in wild-type *Pseudomonas aeruginosa* PAO1. Journal of Antimicrobial Chemotherapy 51, 991–994.

Morita Y, Tomida J, Kawamura Y. (2012a) Primary mechanisms mediating aminoglycoside resistance in the multidrug-resistant *Pseudomonas aeruginosa* clinical isolate PA7. Microbiology 158, 1071–1083.

Morita Y, Tomida J, Kawamura Y. (2012b) MexXY multidrug efflux system of *Pseudomonas aeruginosa*. Frontiers in Microbiology 3, 408.

Morita Y, Tomida J, Kawamura Y. (2015) Efflux-mediated fluoro- quinolone resistance in the multidrug-resistant *Pseudomonas aeruginosa* clinical isolate PA7:identification of a novel MexS variant involved in upregulation of the mexEF-oprN multidrug efflux operon. Frontiers in Microbiology 6, 8.

Mousa JJ, Bruner SD. (2016) Structural and mechanistic diversity of multidrug transporters. Natural Product Reports 33, 1255–1267.

Munita JM, Arias CA. (2016) Mechanisms of Antibiotic Resistance. Microbiology Spectrum, 4.

Murugan N, Malathi J, Umashankar V, Madhavan HN. (2014) Comparative Genomic Analysis of Multidrug-Resistant *Pseudomonas aeruginosa* Clinical Isolates VRFPA06 and VRFPA08 with VRFPA07. Genome Announcements 2, e00140–14.

Murugan N, Malathi J, Umashankar V, Madhavan HN. (2016) Unraveling genomic and phenotypic nature of multidrug-resistant (MDR) *Pseudomonas aeruginosa* VRFPA04 isolated from keratitis patient. Microbiological Research 193, 140–149.

NAP-AMR. (2017) National Action Plan on Antimicrobial Resistance. Government of India.

Narayanan N, Suresh M, Rajamma JP, Ramakrishnan MKP. (2016) Detection of *bla*NDM-1 and genetic relatedness in clinical isolates of *Escherichia coli* producing extended spectrum *β*-lactamase from tertiary care centres in South India. Advances in Microbiology 6, 125-132.

Nies DH. (2003) Efflux-mediated heavy metal resistance in prokaryotes. FEMS Microbiology Reviews 27, 313–339.

Nikaido H, Pagès J-M. (2012) Broad specificity efflux pumps and their role in multidrug resistance of gram negative bacteria. FEMS microbiology reviews 36, 340-363.

Nithya N, Remitha R, Jayasree PR, Faisal M, Manish KPR. (2017) Analysis of beta-lactamases, $bla_{\text{NDM-1}}$phylogeny & plasmid replicons in multidrug-resistant *Klebsiella* spp. from a tertiary care centre in south India. Indian Journal of Medical Research 146, 38-45.

Ocampo-Sosa AA, Cabot G, Rodríguez C, Roman E, Tubau F, Macia MD, Moya B, Zamorano L, Suárez C, Peña C, Domínguez MA, Moncalián G, Oliver A, Martínez-Martínez L; Spanish Network for Research in Infectious Disease.

(2012) Alterations of OprD in carbapenem-intermediate and -susceptible strains of *Pseudomonas aeruginosa* isolated from patients with bacteremia in a Spanish multicenter study. Antimicrobial Agents Chemotherapy 56, 1703–1713.

Ochs MM, Lu CD, Hancock REW, Abdelal AT. (1999) Amino acid-mediated induction of the basic amino acid-specific outer membrane porin OprD from *Pseudomonas aeruginosa*. Journal of Bacteriology 181, 5426–5432.

Pan YP, Xu YH, Wang ZX, Fang YP, Shen JL. (2016) Overexpression of MexAB-OprM efflux pump in carbapenem resistant *Pseudomonas aeruginosa*. Archives of Microbiology 198, 565-571.

Paphitou NI. (2013) Antimicrobial resistance: action to combat the rising microbial challenges. International Journal of Antimicrobial Agents 42, S25–S28.

Pavel A, Vasile C. (2012) PyElph – a software tool for gel images analysis and phylogenetics. BMC Bioinformatics 13.

Perron K, Caille O, Rossier C, Van Delden C, Dumas JL, Köhler T. (2004) CzcR-CzcS, a two-component system involved in heavy metal and carbapenem resistance in *Pseudomonas aeruginosa*. Journal of Biological Chemistry 279, 8761–8768.

Pfaffl MW. (2001) A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Research 29, e45.

Piddock LJV. (2006a) Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. Clinical Microbiology Reviews 19, 382-402.

Piddock LJV. (2006b) Multidrug-resistance efflux pumps – not just for resistance. Nature Reviews 4, 629-636.

Pirnay JP, De Vos D, Mossialos D, Vanderkelen A, Cornelis P, Zizi M. (2002) Analysis of the *Pseudomonas aeruginosa* oprD gene from clinical and environmental isolates. Environmental Microbiology 4, 872–882.

Podder MP, Rogers L, Daley PK, Keefe GP, Whitney HG, Tahlan K. (2014) *Klebsiella* species associated with bovine mastitis in Newfoundland. PLoS ONE 9, e106518.

Poole K, Krebes K, McNally C, Neshat S. (1993) Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. Journal of Bacteriology 175, 7363-7372.

Poole K. (2001) Multidrug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms. Journal of Molecular Microbiology and Biotechnology 3, 255–264.

Poole K. (2004) Efflux-mediated multiresistance in gram-negative bacteria. Clinical Microbiology and Infection 10, 12–26.

Poole K. (2008) Bacterial multidrug efflux pumps serve other functions. Microbe 3, 179–185.

Poole K. (2011) *Pseudomonas aeruginosa*: resistance to the max. Frontiers in Microbiology 2, 65.

Poole K. (2012) Stress responses as determinants of antimicrobial resistance in gram-negative bacteria. Trends in Microbiology 20, 227–234.

Poonsuk K, Tribuddharat C, Chuanchuen R. (2014) Simultaneous overexpression of multidrug efflux pumps in *Pseudomonas aeruginosa* non-cystic fibrosis clinical isolates. Canadian Journal of Microbiology 60, 437– 443.

Porras-Gómez M, Vega-Baudrit J, Núñez-Corrales S. (2012) Overview of multidrug-resistant *Pseudomonas aeruginosa* and novel therapeutic approaches. Journal of Biomaterials and Nanobiotechnology 3, 519-527.

Posada D. (2008) jModelTest: phylogenetic model averaging. Molecular Biology and Evolution 25, 1253–1256.

Pourakbari B, Yaslianifard S, Yaslianifard S, Mahmoudi S, Keshavarz-Valian, Mamishi S. (2016) Evaluation of efflux pumps gene expression in resistant *Pseudomonas aeruginosa* isolates in an Iranian referral hospital. Iranian Journal of Microbiology 8, 249–56.

Prestinaci F, Pezzotti P, Pantosti A. (2015) Antimicrobial resistance: a global multifaceted phenomenon. Pathogens and Global Health 109, 309–318.

Purssell A, Fruci M, Mikalauskas A, Gilmour C, Poole K. (2015) EsrC, an envelope stress-regulated repressor of the *mexCD-oprJ* multidrug efflux operon in *Pseudomonas aeruginosa*. Environmental Microbiology 17, 186–198.

Purssell A, Poole K. (2013) Functional characterization of the NfxB repressor of the mexCD–oprJ multidrug efflux operon of *Pseudomonas aeruginosa*. Microbiology 159, 2058–2073.

Putman M, van Veen HW, Konings WN. (2000) Molecular properties of bacterial multidrug transporters. Microbiology and Molecular Biology Reviews 64, 672-693.

Puzari M, Chetia P. (2017) RND efflux pump mediated antibiotic resistance in gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*: a major issue worldwide. World Journal of Microbiology and Biotechnology *33,1–8.*

Quale J, Bratu S, Gupta J, Landman D. (2006) Interplay of efflux system, *ampC*, and *oprD* expression in carbapenem resistance of *Pseudomonas aeruginosa* clinical Isolates. Antimicrobial Agents and Chemotherapy 50, 1633–1641.

Ranaweera I, Shrestha U, Ranjana KC, Kakarla P, Willmon TM, Hernandez AJ, Mukherjee MM, Barr SR, Varela MF. (2015) Structural comparison of

bacterial multidrug efflux pumps of the major facilitator superfamily. Trends in Cell & Molecular Biology 10, 131–140.

Rodrigues L, Ramos J, Couto I, Amaral L, Viveiros M. (2011) Ethidium bromide transport across *Mycobacterium smegmatis* cell-wall: correlation with antibiotic resistance. BMC Microbiology 11, 35.

Rodríguez-Martínez JM, Poirel L, Nordmann P. (2009) Molecular epidemiology and mechanisms of carbapenem resistance in *Pseudomonas aeruginosa*. Antimicrobial Agents and Chemotherapy 53, 4783–4788.

Roy PH, Tetu SG, Larouche A, Elbourne L, Tremblay S, Ren Q, Dodson R, Harkins D, Shay R, Watkins K, Mahamoud Y, Paulsen IT. (2010) Complete genome sequence of the multiresistant taxonomic outlier *Pseudomonas aeruginosa* PA7. PLoS One 5, e8842.

Sabtu N, Enoch DA, Brown NM. (2015) Antibiotic resistance: what, why, where, when and how?. *British Medical Bulletin* 116, 105–113.

Saga T, Yamaguchi K. (2009) History of Antimicrobial Agents and Resistant. Japan Medical Association Journal 137, 103–108.

Sakhtah H, Koyama L, Zhang Y, Morales DK, Fields BL, Price-Whelan A, Hogan DA, Shepard K, Dietrich LEP. (2016) The *Pseudomonas aeruginosa* efflux pump MexGHI-OpmD transports a natural phenazine that controls gene expression and biofilm development. Proceedings of the National Academy of Sciences of the United States of America 113, E3538-47.

Sambrook J, Fritsch EF, Maniatis T. (1989) Molecular cloning: A laboratory manual. 2nd edn. Cold Spring Habor Press Cold Spring Harbor, NY.

Sambrook J, Russell DW. (2001) Molecular cloning: A laboratory manual. 3rd edn. Cold Spring Habor Press Cold Spring Harbor, NY.

Sanbongi Y, Shimizu A, Suzuki T, Nagaso H, Ida T, Maebashi K, Gotoh N. (2009) Classification of OprD sequence and correlation with antimicrobial activity

of carbapenem agents in *Pseudomonas aeruginosa* clinical isolates collected in Japan. Microbiology and Immunology 53, 361-367.

Sandhu R, Dahiya S, Sayal P. (2016) Evaluation of multiple antibiotic resistance (MAR) index and Doxycycline susceptibility of *Acinetobacter* species among inpatients. Indian Journal of Microbiology Research 3, 299-304.

Savli H, Karadenizli A, Kolayli F, Gundes S, Ozbek U, Vahaboglu H. (2003) Expression stability of six housekeeping genes: A proposal for resistance gene quantification studies of *Pseudomonas aeruginosa* by real-time quantitative RT-PCR. Journal of Medical Microbiology 52, 403-408.

Schiavano GF, Carloni E, Andreoni F, Magi S, Chironna M, Brandi G, Amagliani G. (2017) Prevalence and antibiotic resistance of *Pseudomonas aeruginosa* in water samples in central Italy and molecular characterization of oprD in imipenem resistant isolates. PLoS ONE 12, e0189172.

Seeliger D, de Groot BL. (2010) Ligand docking and binding site analysis with PyMOL and Autodock/Vina. Journal of Computer-Aided Molecular Design 24, 417-422.

Sharma A. (2011) Antimicrobial resistance: no action today, no cure tomorrow. Indian Journal of Medical Microbiology 29, 91-92.

Singh AR. (2011) Science, names giving and names calling: Change NDM-1 to PCM. Mens Sana Monographs 9, 294-319.

Singh M, Yau YCW, Wang S, Waters V, Kumar A. (2017) MexXY efflux pump overexpression and aminoglycoside resistance in cystic fibrosis isolates of *Pseudomonas aeruginosa* from chronic infections. Canadian Journal of Microbiology 63, 929-938.

Sit PS, Teh CS, Idris N, Sam IC, Syed Omar SF, Sulaiman H, Thong KL, Kamarulzaman A, Ponnampalavanar S. (2017) Prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) infection and the molecular

characteristics of MRSA bacteraemia over a two-year period in a tertiary teaching hospital in Malaysia. BMC Infectious Disease 17, 274.

Sivaneson M, Mikkelsen H, Ventre I, Bordi C, Filloux A. (2011) Two-component regulatory systems in *Pseudomonas aeruginosa*: an intricate network mediating fimbrial and efflux pump gene expression. Molecular Microbiology 79, 1353–1366.

Soares-Castro P, Marques D, Demyanchuk S, Faustino A, Santos PM. (2011) Draft genome sequences of two *Pseudomonas aeruginosa* clinical isolates with different antibiotic susceptibilities. Journal of Bacteriology 193, 5573.

Sobel ML, Hocquet D, Cao L, Plesiat P, Poole K. (2005) Mutations in *PA3574* (*nalD*) lead to increased MexAB-OprM expression and multidrug resistance in laboratory and clinical isolates of *Pseudomonas aeruginosa*. Antimicrobial Agents and Chemotherapy 49, 1782–1786.

Sobel ML, McKay GA, Poole K. (2003) Contribution of the MexXY multidrug transporter to aminoglycoside resistance in *Pseudomonas aeruginosa* clinical Isolates. Antimicrobial Agents and Chemotherapy 47, 3202–3207.

Sobel ML, Neshat S, Poole K. (2005) Mutations in PA2491 (*mexS*) promote MexT-dependent *mexEF-oprN* expression and multidrug resistance in a clinical strain of *Pseudomonas aeruginosa*. Journal of Bacteriology 187, 1246–1253.

Spengler G, Kincses A, Gajdacs M, Amaral L. (2017) New roads leading to old destinations: efflux pumps as targets to reverse multidrug resistance in bacteria. Molecules 22, E468.

Strateva T, Yordanov D. (2009) *Pseudomonas aeruginosa*–a phenomenon of bacterial resistance. Journal of Medical Microbiology 58, 1133–1148.

Suman G, Khan M, Sabitha K, Jamil K. (2006) Mutation in mexR-gene leading to drug resistance in corneal keratitis in human. Indian Journal of Experimental Biology 44, 929–936.

Sun J, Deng Z, Yan A. (2014) Bacterial multidrug efflux pumps: mechanisms, physiology and pharmacological exploitations. Biochemical and Biophysical Research Communications 453, 254–267.

Symmons MF, Bokma E, Koronakis E, Hughes C, Koronakis V. (2009) The assembled structure of a complete tripartite bacterial multidrug efflux pump. Proceedings of the National Academy of Sciences of the United States of America 106, 7173–7178.

TB India. (2017) Revised national tuberculosis control programme, Annual status report. Central TB division, Government of India.

Tello A, Austin B, Telfer TC. (2012) Selective pressure of antibiotic pollution on bacteria of importance to public health. Environmental Health Perspectives 120, 1100-1106.

Tenover FC. (2006) Mechanisms of antimicrobial resistance in bacteria. The American journal of medicine 119, S3-S10.

Tian Z-X, Yi X-X, Cho A, O'Gara F, Wang Y-P. (2016) CpxR activates MexAB-OprM efflux pump expression and enhances antibiotic resistance in both laboratory and clinical *nalB*-type isolates of *Pseudomonas aeruginosa*. PLoS Pathogens 12, e1005932.

Vaez H, Faghri J, Isfahani BN, Moghim S, Yadegari S, Fazeli H, Moghofeei M, Safaei HG. (2014) Efflux pump regulatory genes mutations in multidrug resistance *Pseudomonas aeruginosa* isolated from wound infections in Isfahan hospitals. Advanced Biomedical Research 3, 117.

Van Acker H, Gielis J, Acke M, Cools F, Cos P, Coenye T. (2016) The Role of Reactive Oxygen Species in Antibiotic-Induced Cell Death in *Burkholderia cepacia* complex bacteria. PLoS ONE 11, e0159837.

van Belkum A, Soriaga LB, LaFave MC, Akella S, Veyrieras J-B, Barbu EM, Shortridge D, Blanc B, Hannum G, Zambardi G, Miller K, Enright MC, Mugnier N, Brami D, Schicklin S, Felderman M, Schwartz AS, Richardson

TH, Peterson TC, Hubby B, Cady KC. (2015) Phylogenetic distribution of CRISPR-Cas systems in antibiotic-resistant *Pseudomonas aeruginosa.* mBio 6, e01796-15.

van Hoek AH, Mevius D, Guerra B, Mullany P, Roberts AP, Aarts HJM. (2011) Acquired antibiotic resistance genes: an overview. Frontiers in Microbiology 2.

Vargiu AV, Pos KM, Poole K, Nikaido H. (2016) Editorial: Bad bugs in the XXIst century: resistance mediated by multi-drug efflux pumps in gram-negative bacteria. Frontiers in Microbiology 7, 833.

Ventola CL. (2015) The antibiotic resistance crisis: part 1: causes and threats. Pharmacy and Therapeutics 40, 277-283.

Vincze T, Posfai J, Roberts RJ. (2003) NEBcutter: a program to cleave DNA with restriction enzymes. Nucleic Acids Research 31, 3688-3691.

von Wintersdorff CJH, Penders J, van Niekerk JM, Mills ND, Majumder S, van Alphen LB, Savelkoul PHM and Wolffs PFG. (2016) Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. Frontiers in Microbiology 7, 173.

Webber MA, Piddock LJV. (2003) The importance of efflux pumps in bacterial antibiotic resistance. Journal of Antimicrobial Chemotherapy 51, 9–11.

Westfall LW, Carty NL, Layland N, Kuan P, Colmer-Hamood JA, Hamood AN. (2006) *mvaT* mutation modifies the expression of the *Pseudomonas aeruginosa* multidrug efflux operon *mexEF-oprN*. FEMS Microbiology Letters 255, 247–254.

WHO. (2010) Prevention and containment of antimicrobial resistance. Report of a regional meeting Chiang Mai, Thailand, 8th to 11th of June.

WHO. (2017a) Global priority list of antibiotic-resistant bacteria to guide research, discovery and development of new antibiotics.

WHO. (2017b) Guidelines for the prevention and control of carbapenem-resistant *Enterobacteriaceae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* in health care facilities. Geneva: Licence: CC BY-NC-SA 3.0 IGO.

WHO. (2018a) http://www.who.int/mediacentre/news/releases/2018/antibiotic-resistance-found/en/ cited on 29.01.2018.

WHO. (2018b) http://www.who.int/mediacentre/factsheets/fs104/en/ cited on 08.02.2018.

WHO-Govt. of India Ministry of Health and Family Welfare Report. (2016) Antimicrobial resistance and its containment in India, Inter-Ministerial Review Meeting on Antimicrobial Resistance.

Wilke MS, Heller M, Creagh AL, Haynes CA, McIntosh LP, Poole K, Strynadka N C. (2008) The crystal structure of MexR from *Pseudomonas aeruginosa* in complex with its antirepressor ArmR. Proceedings of the National Academy of Sciences of the United States of America 105, 14832–14837.

Winsor GL, Griffiths EJ, Lo R, Dhillon BK, Shay JA, Brinkman FSL. (2016) Enhanced annotations and features for comparing thousands of *Pseudomonas* genomes in the *Pseudomonas* genome database. Nucleic Acids Research 44(Database issue), D646-D653.

Wolter DJ, Hanson ND, Lister PD. (2004) Insertional inactivation of *oprD* in clinical isolates of *Pseudomonas aeruginosa* leading to carbapenem resistance. FEMS Microbiology Letters 236, 137–143.

Wright EA, Di Lorenzo V, Trappetti C, Liciardi M, Orru G, Viti C, Bronowski C, Hall AJ, Darby AC, Oggioni MR, Winstanley C. (2015) Divergence of a strain of *Pseudomonas aeruginosa* during an outbreak of ovine mastitis. Veterinary Microbiology 175, 105–113.

Wright GD. (2005) Bacterial resistance to antibiotics: enzymatic degradation and modification. Advanced Drug Delivery Reviews 57, 1451–1470.

Xavier DE, Picão RC, Girardello R, Fehlberg LC, Gales AC. (2010) Efflux pumps expression and its association with porin down-regulation and β-lactamase production among *Pseudomonas aeruginosa* causing bloodstream infections in Brazil. BMC Microbiology 10, 217.

Yoneda K, Chikumi H, Murata T, Gotoh N, Yamamoto H, Fujiwara H, Nishino T, Shimizu E. (2005) Measurement of *Pseudomonas aeruginosa* multidrug efflux pumps by quantitative real-time polymerase chain reaction. FEMS Microbiology Letters 243, 125–131.

Yong D, Lee K, Yum JH, Shin HB, Rossolini GM, ChongY. (2002) Imipenem–EDTA disc method for differentiation of metallo –beta-lactamase producing clinical isolates of *Pseudomonas* spp. and *Acinetobacter* spp. Journal of Clinical Microbiology 40, 3798–3801.

Zaman SB, Hussain MA, Nye R, Mehta V, Mamun KT, Hossain N. (2017) A review on antibiotic resistance: alarm bells are ringing. Cureus 9, e1403.

Zavascki AP, Carvalhaes CG, Picão RC, Gales AC. (2010) Multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii*: resistance mechanisms and implications for therapy. Expert Review of Anti-Infective Therapy 8, 71–93.

Zeng ZR, Wang WP, Huang M, Shi LN, Wang Y, Shao HF. (2014) Mechanisms of carbapenem resistance in cephalosporin- susceptible *Pseudomonas aeruginosa* in China. Diagnostic Microbiology and Infectious Disease 78, 268–270.

Zgurskaya HI, Löpez CA, Gnanakaran S. (2015) Permeability barrier of gram-negative cell envelopes and approaches to bypass it. ACS infectious diseases 1, 512-522.

Ziha-Zarifi I, Llanes C, Köhler T, Pechere J-C, Plesiat P. (1999) In vivo emergence of multidrug-resistant mutants of *Pseudomonas aeruginosa* overexpressing the active efflux system MexA-MexB-OprM. Antimicrobial Agents and Chemotherapy 43, 287–291.

APPENDIX

Bacteriological Media

Luria Bertani (LB) Media:

Trypton (w/v)	10g
NaCl (w/v)	10g
Yeast Extract (w/v)	5g
Water to one litre	
For LB agar added 2% (w/v) agar	

Other culture media:

(1) McConkey agar

(2) Mueller-Hinton agar (susceptibility tests)

(3) Simmon citrate medium (citrate utilization test)

(4) Mannitol motility media (mannitol fermentation and motility assessment)

All these media were purchased from Himedia, Mumbai.

Buffers, solutions and reagents

(All the reagents used were either Analytical reagent/ Molecular Biology grade)

CIA (Chloroform Isoamyl alcohol):

Chloroform	24 parts
Isoamyl alcohol	1 part

Crystal violet solution:

Crystal violet	0.5%

CTAB/NaCl:

Cetyl-trimethyl ammonium bromide	10g
NaCl	4.1g
Water to 100 ml	

6x Gel loading buffer

Bromophenol blue	0.25%
xylene cyanol FF	0.25%
glycerol in water	30%
Stored at $4^{o}C$	

GTE (Glucose-Tris-EDTA):

Glucose	50 Mm
Tris-Cl (pH 8.0)	25 Mm
EDTA (pH 8.0)	10 Mm
Autoclaved and stored at $4^{o}C$	

Kovac's reagent:

p - Dimethylaminobenzaldehyde	10 g
Isoamyl or isobutyl alcohol	150 ml
Conc. HCl	50 ml

McFarland Standard (0.5):

1.175% barium chloride dihydrate ($BaCl_2$. $2H_2O$)	0.05 ml
1% H_2SO_4	9.95 ml

Methyl red reagent:

Methyl red	0.1g
Ethyl alcohol (95%)	300 ml
water	200 ml

Phenol:CIA:

Equilibrated phenol (pH 8.0)	1part
CIA	1 part

Potassium acetate:

5 M potassium acetate	60 ml
Glacial acetic acid	11.5 ml
Water	28.5 ml

Safranin solution:

Safranin	0.5%

Solution II (Plasmid isolation):

NaOH (Freshly diluted from stock)	0.1 N
SDS	1%

STE (Sodium Chloride-Tris-EDTA):

NaCl	0.1 M
Tris-Cl (pH 8.0)	10 mM
EDTA (pH 8.0)	1mM
Prepared and autoclaved	

5x Tris Borate EDTA (TBE) – pH 8.0:

Tris base	54g
Boric acid	27.5g
0.5 M EDTA (pH 8.0)	20 ml
Water to one litre	

Tris EDTA (TE) – pH 8.0:

Tris-Cl (pH 8.0)	10 mM
EDTA (pH 8.0)	1 mM

Voges-Proskauer reagent:

VP Reagent A – α-Naphthol in ethyl alcohol.

VP Reagent B – 40% KOH

PUBLICATIONS (PEER - REVIEWED) FROM THE DOCTORAL THESIS

Manju Suresh, Nithya N, Jayasree PR, Manish Kumar PR. Detection and prevalence of efflux pump-mediated drug resistance in clinical isolates of multidrug-resistant gram-negative bacteria from North Kerala, India. ***Asian Journal of Pharmaceutical and Clinical Research*** 9(3): 324–327, 2016 (**Innovare Academic Sciences**; Impact factor: **IPP-0.588**).

Manju Suresh, *Nithya N,* Jayasree PR, Vimal KP, Manish Kumar PR. Mutational analyses of regulatory genes, *mexR, nalC, nalD* and *mexZ* of *mexAB-oprM* and *mexXY* operons, in efflux pump hyperexpressing multidrug-resistant clinical isolates of *Pseudomonas aeruginosa*. ***World Journal of Microbiology and Biotechnology*** 34: 83, 2018 (**Springer Nature**; Impact factor: **Thomson Reuters – 2.1**).

Manju Suresh, Sinosh Skariyachan, Nithya N, Jayasree PR, Manish Kumar PR. Mutational variations, restriction fragment length polymorphism in *oprD* porin gene and molecular modelling of the mutant protein in multidrug resistant *Pseudomonas aeruginosa* (**communicated**).

Manju Suresh, Nithya N, Jayasree PR, Manish Kumar PR. Mutational and phylogenetic analysis of *nfxB* gene in multidrug-resistant clinical isolates of *Pseudomonas aeruginosa* hyperexpressing MexCD-OprJ efflux pump (**communicated**).

CONFERENCE PAPERS

Manju Suresh, Nithya N, Dr. P.R. Jayasree, Dr. P.R. Manish Kumar. Prevalence and RAPD Analysis of various beta-lactamases - ESBL, AmpC and MBL - among multidrug resistant clinical isolates of *Pseudomonas aeruginosa* from North Kerala. 28th Kerala Science Congress held at University of Calicut. 28-30 January, 2016.

Manju Suresh, Jayasree P. R, Manish Kumar P. R. Prevalence of MexAB-OprM and MexXY efflux systems in multidrug- resistant clinical isolates of *Pseudomonas aeruginosa*: A threat to environment. Indian Science Congress Association (ISCA) Coimbatore chapter held at Kongunadu arts and science college, Coimbatore. 09-11 October 2017 (**Best paper award**).

Manju Suresh , Jayasree P. R. & Manish Kumar P. R. Contribution of porins and beta-lactamases in carbapenem resistance among multidrug resistant *Pseudomonas aeruginosa* and their genetic relatedness. 27th Swadeshi Science Congress held at Amrita Viswa Vidyapeetham, Amrita University, Kollam. 07-09 November 2017.

OTHER RELATED PUBLICATION

Nithya Narayanan, **Manju Suresh**, Jayasree Pullampara Rajamma, Manish Kumar Panickassery Ramakrishnan. Detection of blaNDM-1 and genetic relatedness in clinical isolates of *Escherichia coli* producing extended spectrum β-lactamase from tertiary care centres in South India. ***Advances in Microbiology*** 6(3): 125-132,

2016 (**Scientific Research Publishing**; Impact Factor: **Google-based Journal - 1.07**)

ACCESSION NUMBERS OF DNA SEQUENCES DEPOSITED IN GENBANK , NCBI

GenBank accession numbers – 14

Manju,S., Nithya,N., Jayasree,P.R. and Manish Kumar,P.R. (2018) *Pseudomonas aeruginosa* strain Pa6 NalD (nalD) gene, complete cds. GenBank accession no. MG744560.

Manju,S., Nithya,N., Jayasree,P.R. and Manish Kumar,P.R. (2018) *Pseudomonas aeruginosa* strain Pa13 NalD (nalD) gene, complete cds. GenBank accession no. MG757449.

Manju,S., Nithya,N., Jayasree,P.R. and Manish Kumar,P.R. (2018) *Pseudomonas aeruginosa* strain Pa16 NalD (nalD) gene, complete cds. GenBank accession no. MG757450.

Manju,S., Nithya,N., Jayasree,P.R. and Manish Kumar,P.R. (2018) *Pseudomonas aeruginosa* strain Pa6 NalC (nalC) gene, complete cds. GenBank accession no. MG757451.

Manju,S., Nithya,N., Jayasree,P.R. and Manish Kumar,P.R. (2018) *Pseudomonas aeruginosa* strain Pa13 NalC (nalC) gene, complete cds. GenBank accession no. MG757452.

Manju,S., Nithya,N., Jayasree,P.R. and Manish Kumar,P.R. (2018) *Pseudomonas aeruginosa* strain Pa16 NalC (nalC) gene, partial cds. GenBank accession no. MG757453.

Manju,S., Nithya,N., Jayasree,P.R. and Manish Kumar,P.R. (2018) *Pseudomonas aeruginosa* strain Pa6 MexR (mexR) gene, partial cds. GenBank accession no. MG757454.

Manju,S., Nithya,N., Jayasree,P.R. and Manish Kumar,P.R. (2018) *Pseudomonas aeruginosa* strain Pa13 MexR (mexR) gene, partial cds. GenBank accession no. MG757455.

Manju,S., Nithya,N., Jayasree,P.R. and Manish Kumar,P.R. (2018) *Pseudomonas aeruginosa* strain Pa16 MexR (mexR) gene, complete cds. GenBank accession no. MG757456.

Manju,S., Nithya,N., Jayasree,P.R. and Manish Kumar,P.R. (2018) *Pseudomonas aeruginosa* strain Pa5 MexZ (mexZ) gene, complete cds. GenBank accession no. MG757457.

Manju,S., Nithya,N., Jayasree,P.R. and Manish Kumar,P.R. (2018) *Pseudomonas aeruginosa* strain Pa25 MexZ (mexZ) gene, complete cds. GenBank accession no. MG757458.

Manju,S., Nithya,N., Jayasree,P.R. and Manish Kumar,P.R. (2018) *Pseudomonas aeruginosa* strain Pa29 MexZ (mexZ) gene, partial cds. GenBank accession no. MG757459.

Manju,S., Nithya,N., Jayasree,P.R. and Manish Kumar,P.R. (2018) *Pseudomonas aeruginosa* strain Pa16 MexZ (mexZ) gene, partial cds. GenBank accession no. MG757460.

Manju,S., Nithya,N., Jayasree,P.R. and Manish Kumar,P.R. (2018) *Pseudomonas aeruginosa* strain Pa13 MexZ (mexZ) gene, partial cds. GenBank accession no. MG757461.

ACCESSION NUMBERS OF DNA SEQUENCES DEPOSITED IN GENBANK, NCBI (YET TO BE RELEASED)

GenBank accession numbers –53

The *oprD* gene sequences of MDR *P. aeruginosa* : MH122946-MH122954, MH135303-MH135311, MH142581-MH142588 (**26 nos.**)

The *nfxB* gene sequences of MDR *P. aeruginosa* : MH346508 - MH346510 (**3 nos.**)

The *mexT* gene sequences of MDR *P. aeruginosa* : MH397275-MH397298 (**24 nos.**)

OTHER RELATED ACCESSION NUMBERS OF DNA SEQUENCES DEPOSITED IN GENBANK , NCBI

GenBank accession numbers - 9

Nithya,N., **Manju,S**., Jayasree,P.R. and Kumar,P.R.M. (2016) *Escherichia coli* strain E7 New Delhi metallo-beta-lactamase (blaNDM) gene, blaNDM-1 allele, partial cds. GenBank accession no. KX100581.

Nithya,N., **Manju,S**., Jayasree,P.R. and Kumar,P.R.M. (2016) *Escherichia coli* strain E8 New Delhi metallo-beta-lactamase(blaNDM) gene, partial cds. GenBank accession no. KX100582.

Nithya,N., **Manju,S.**, Jayasree,P.R. and Kumar,P.R.M. (2016) *Escherichia coli* strain E10 New Delhi metallo-beta-lactamase(blaNDM) gene, partial cds. GenBank accession no. KX100583.

Nithya,N., **Manju,S**., Jayasree,P.R. and Kumar,P.R.M. (2016) *Escherichia coli* strain E23 New Delhi metallo-beta-lactamase(blaNDM) gene, partial cds. GenBank accession no. KX100584.

Nithya,N., Remitha,R., **Manju,S**., Jayasree,P.R. and Kumar,P.R.M. (2016) *Escherichia coli* strain EGE14 class 1 integron, partial sequence. GenBank accession no. KU751767.

Nithya,N., Remitha,R., **Manju,S.,** Jayasree,P.R. and Kumar,P.R.M. (2016) *Escherichia coli* strain EGE20 class 1 integron, partial sequence. GenBank accession no. KU751768.

Nithya,N., Remitha,R., **Manju,S**., Jayasree,P.R. and Kumar,P.R.M. (2016) *Klebsiella sp*. EGE5 class 1 integron, partial sequence. GenBank accession no. KU751769.

Nithya,N., Remitha,R., **Manju,S**., Jayasree,P.R. and Kumar,P.R.M. (2016) *Escherichia coli* strain EGE25 class 1 integron, partial sequence. GenBank accession no. KU751770.

Nithya,N., Remitha,R., **Manju,S**., Jayasree,P.R. and Kumar,P.R.M. (2016) *Escherichia coli* strain EGE26 class 1 integron, partial sequence. GenBank accession no. KU751771.