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CHROMOSOMAL ANTIBIOTIC RESISTANCE MECHANISMS IN *PSEUDOMONAS AERUGINOSA* AND *NEISSERIA GONORRHOEAE*

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To my parents

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ABSTRACT

The progressive increase in fluoroquinolone resistant *N. gonorrhoeae* and emergence of multiple antibiotic resistant *P. aeruginosa* are growing concerns among physicians and health policy makers. In *N. gonorrhoeae* chromosomal gene mutations encoding different subunits of DNA gyrase and topoisomerase IV have been considered the main mechanism of fluoroquinolone resistance even though these changes do not explain the varying MICs of resistant strains. Whereas in *P. aeruginosa* the MexXY efflux pump is described as the predominant manifestation of aminoglycoside resistance in isolates from cystic fibrosis lungs, the aminoglycoside modifying enzymes (AME) contribute to resistance in *P. aeruginosa* strains isolated from other infections. Modification of target (16s rRNA) also accounted for several cases of aminoglycoside resistance but has not been investigated in clinical isolates. The main mechanism of carbapenem (meropenem and imipenem) resistance in *P. aeruginosa* is down regulation of porin protein OprD and/or increased efflux by MexAB-OprM. Alterations of penicillin binding proteins have also been accounted for increased resistance to carbapenems in clinical isolates of *P. aeruginosa.*

The major focus of this thesis is to study the chromosomally mediated antibiotic resistance mechanisms of fluoroquinolones in *Neisseria gonorrhoeae* and aminoglycosides and carbapenems in *Pseudomonas aeruginosa*. To assess fluoroquinolone resistance mechanisms in *N. gonorrhoeae* we transformed bacterial DNA from clinically resistant strains to sensitive strain and studied the involvement of chromosomal genes *gyrA*, *parE*, *porB1b* and *lysR* by PCR and sequencing. A total of 40 cystic fibrosis isolates of *P. aeruginosa* were included in this study to understand the aminoglycoside and in particular the amikacin resistance mechanism in CF environment. An array of chromosomal determinants, which might have role in aminoglycoside resistance including *mexY*, *mexB*, *oprM*, *oprD*, *mexZ, aph (3′)-IIps*, PA5471, *galU*, *rplY* and genes involved in electron transport chains were analyzed by sequencing or real time PCR. We examined 16S rRNA A-site by pyrosequencing of some clinically relevant strains where other mechanisms failed to explain aminoglycoside resistance properly. To assess the carbapenem resistance mechanism, conjugation experiments have been performed between resistant clinical strains and a laboratory strain of *P. aeruginosa*. The transconjugants with low susceptibility to carbapenems were further studied for the expression and sequence of OprD by realtime PCR and presence of mutations in different hotspots of penicillin binding protein genes by sequencing.

We have concluded that alteration in GyrA subunit of DNA gyrase is the main determinant of fluoroquinolone resistance in *N. gonorrhoeae*. Our study suggests that introduction of additional mutations in *gyrA* and/or *parE* as well as alterations of *porB1b* contribute to ciprofloxacin resistance. In *P. aeruginosa,* clinical strains and transconjugants, we found that downregulation of OprD porin protein is the main mechanism for carbapenem resistance. Many cystic fibrosis patients are infected with *P. aeruginosa* in their early age and generally the same strains persist and colonize the CF lungs over the period of their lifetime. In most cases progenies with different phenotype of the same strains perpetuate in CF lung. We focused on changes in chromosomal determinants of aminoglycoside resistance in CF *P. aeruginosa* isolates. The major chromosomal changes in our study are in the regulatory genes for the efflux pump MexXY followed by the overexpression of pump protein MexY as the dominating mechanism of aminoglycoside resistance in CF *P. aeruginosa* isolates.

LIST OF PUBLICATIONS

I. Lindbäck E, **Islam S**, Unemo M, Lang C, Wretlind B.
Transformation of ciprofloxacin-resistant *Neisseria gonorrhoeae* *gyrA*, *parE* and *porB1b* genes
International Journal of Antimicrobial Agents, 28: 206–211, 2006

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

III. Farra A, **Islam S**, Strålfors A, Sörberg M, Wretlind B
Role of outer membrane protein OprD and penicillin-binding proteins in *Pseudomonas aeruginosa* resistance to imipenem and meropenem
Accepted in International Journal of Antimicrobial Agents

IV. **Islam S**, Oh H, Jalal S, Karpati F, Ciofu O, Høiby N, Wretlind B
Chromosomal resistance mechanism for aminoglycoside resistance in P*seudomonas aeruginosa* cystic fibrosis strains
Submitted in Clinical Microbiology and Infection

CONTENTS

LIST OF ABBREVIATIONS

AAC	Aminoglycoside acetyltransferase
AGIR	Aminoglycoside impermeability-type resistance
AME	Aminoglycoside modifying enzyme
ANT	Aminoglycoside nucleotidyltransferase
APH	Aminoglycoside phosphotransferase
CD	Cluster of Differentiation
CDC	Center for Disease Control and Prevention
CF	Cystic fibrosis
CFPA	Cystic Fibrosis *Pseudomonas aeruginosa*
CFTR	Cystic fibrosis transmembrane conductance regulator
Cp	Crossing point
DHP-1	Dehydropeptidase-1
ESBL	Extended spectrum ß-lactamase
FQ	Fluoroquinolone
GC	Gonococcus
HMW	High Molecular Weight
LMW	Low Molecular Weight
LOS	Lipooligsaccharide
LPS	Lipopolysaccharide
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
Mtr	Multiple transferable resistance
NADH	Nicotinamide Adenine di-nucleotide plus Hydrogen
nt	Nucleotide
PBP	Penicillin binding protein
PFGE	Pulsed field gel electrophoresis
RND	Resistance nodulation and cell division
rRNA	Ribosomal RNA
SMI	Smittskyddsinstitutet
spp.	Species
tRNA	Transfer RNA
TTSS	Type III secretion system
WHO	World Health Organization

Amino Acid Codes

G Glycine Gly	**F** Phenylalanine Phe	**S** Serine Ser	**H** Histidine His
P Proline Pro	**W** Tryptophan Trp	**V** Valine Val	**R** Arginine Arg
A Alanine Ala	**K** Lysine Lys	**I** Isoleucine Ile	**N** Asparagine Asn
L Leucine Leu	**Q** Glutamine Gln	**C** Cysteine Cys	**D** Aspartic Acid Asp
M Methionine Met	**E** Glutamic Acid Glu	**Y** Tyrosine Tyr	**T** Threonine Thr

1 INTRODUCTION

1.1 A BRIEF HISTORY OF ANTIBIOTICS AND EMERGENCE OF RESISTANCE

Bacterial resistance to antibiotics has become one of the main public health concerns world-wide. Since the discovery of penicillin and sulphonamides in early 1900s and their introduction to therapeutic use in 1940s, use of antibiotic were stunning remedies to cure deadly infections and became known as 'miracle drugs'. The quest of finding new drugs introduced new antibiotics into the pipeline and was followed by the introduction of streptomycin, tetracyclines, chloramphenicol and macrolides in the 1950s; and later by trimethoprim and quinolones. From their introduction, antibiotics have been used not only to cure pneumonia, sepsis and other serious diseases but also extensively used to save immunocompromised patients from developing infections and even used to treat stomach ulcers. Unfortunately, antibiotics are frequently prescribed for trivial infections, usually caused by viruses. Large amounts of antibiotics were also used in agriculture. Approximately in 50 years of antibiotic era, about one million tons of antibiotics have been produced, used and disseminated. This flourishing use of antibiotic in so many years ironically led antibiotic to a threatened category of drugs.

Bacterial resistance to antibiotics occurs at low level in natural populations as antibiotics have been produced by some subsets of bacteria or fungi to act on their neighbours. Thus, evolutionary pressure acts on the bacteria attacked by antibiotics to contrive resistance mechanisms through selection of adaptable mutations to survive and contribute to low level of resistance. Excessive use of a new drug will increase the evolutionary pressure to drive low frequency antibiotic resistance in natural bacterial populations to high prevalence and to rapid acquisition of new resistance traits. Antibiotic resistance also depends on the availability of mechanisms of resistance in bacteria. Most of the various resistance mechanisms in pathogenic bacteria seem to be acquired by transmission of genes between bacterial species i.e. from antibiotic producing bacteria or other bacteria that already had developed resistance mechanism to antibiotics [1]. Bacteria have demonstrated their enormous genetic flexibility in avoiding, withstanding or repelling the antibiotic treatment by becoming resistant to one antibiotic after another. The first year of deployment and the dates of detection of clinically significant resistance for some of the antibiotics of major importance in antibiotic therapy are shown in ***Table 1***. After 1970s the number of new antibiotics entering the therapeutic pipeline began to decrease. The discovery and development are expensive, especially considering the speed with which bacterial resistance can arise, it is necessary to understand the mechanism of antibiotic resistance not only to aid better treatment regimen but also to develop new antibiotics as well as the best use of available antibiotics.

The basic mechanisms of major antimicrobial agents on the bacterial cells are shown in ***Figure 1***.

Table 1. *Emergence of antibiotic resistance*

Antibiotics	Year of deployment	Resistance observed
Sulfonamides	1930s	1940s
Penicillin	1943	1946
Streptomycin	1943	1959
Chloramphenicol	1947	1959
Tetracycline	1948	1953
Erythromycin	1952	1988
Vancomycin	1956	1988
Methicillin	1960	1961
Ampicillin	1961	1973
Cephalosporins	1960s	late 1960s

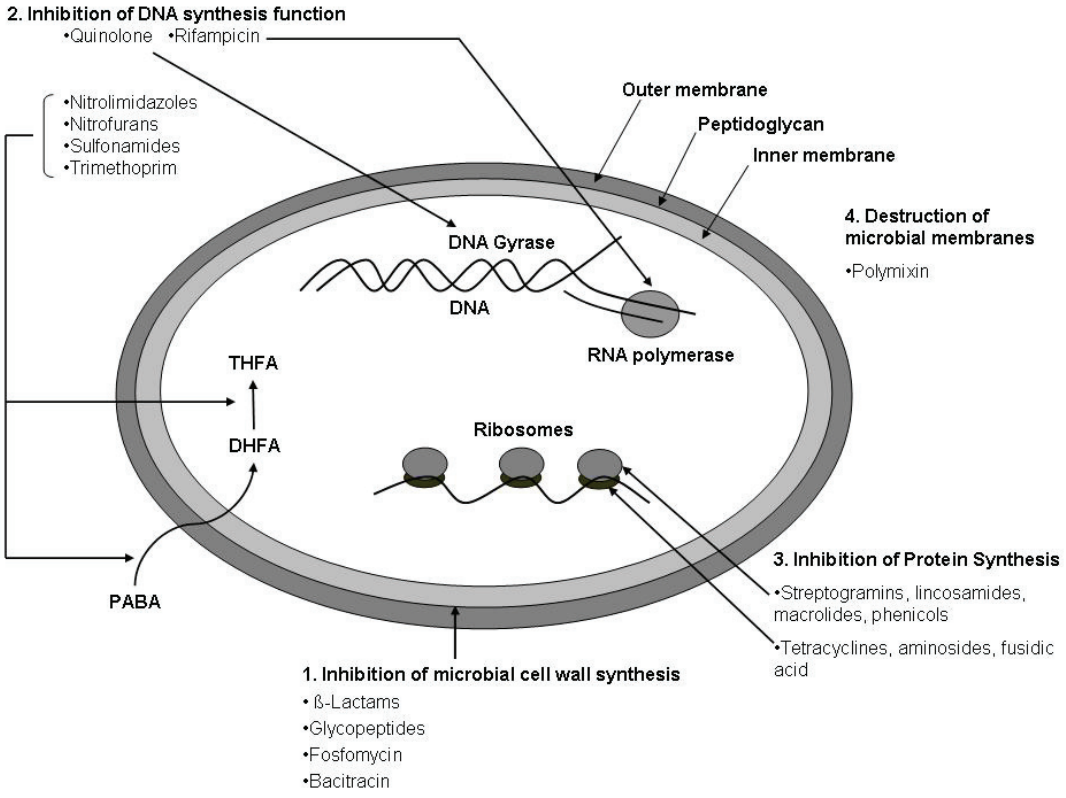

Figure 1. *Mechanisms of action for the most important group of antibiotics*

1.2 QUINOLONES

The quinolones are a group of synthetic antibiotics, first discovered in 1962 as a progenitor, 1,8-naphthyridine, nalidixic acid in the course of carrying out a synthesis of chloroquine. The use of nalidixic acid was limited to the treatment of Gram-negative urinary tract infections. Further development of the structure–activity relationships in the quinolone class has resulted in a large number of new quinolones, and many of which have been advanced to clinical practices.

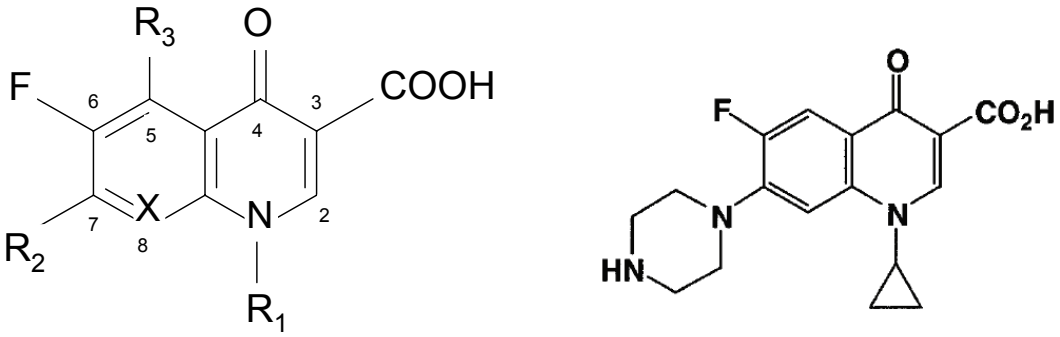

Figure 2. a) *Structure of the quinolone nucleus* ***b)*** *Chemical structure of ciprofloxacin*

The molecular structures of the quinolones have been adapted over time in association with clinical need followed by different substitutions at positions 1, 5, 7 and 8 with good antimicrobial activity (***Figure 2a***). The first generations 4-quinolones, oxolinic acid and cinoxacin (was introduced by fusing additional rings at the 6- and 7-positions) displayed improved Gram-negative activity compared to nalidixic acid, with no or little against Gram-positive cocci, *Pseudomonas aeruginosa*, and anaerobes. The breakthrough came in the quinolone research in 1980s with the introduction of a fluorine molecule at position 6 and a diamine ring at position 7 yielded norfloxacin, the 2^{nd} generation quinolone with substantial Gram-negative and Gram-positive antibacterial activity [2]. Later on ciprofloxacin was formulated by replacing the ethyl group at position 1 by a cyclopropyl group. (***Figure 2b***). Afterwards levofloxacin, sparfloxacin (3^{rd} generation), trovafloxacin, moxifloxacin (4^{th} generation) came into clinical practices with species specific activities.

1.2.1 Mode of actions of quinolones

Quinolones exert their bactericidal activity by acting on two type II topoisomerases i.e. DNA gyrase and topoisomerase IV. Both of these enzymes are A2B2 type of tetrameric enzymes with pairs of two different subunits. The GyrA and GyrB subunits of DNA gyrase are homologous to the ParC and ParE subunits of topoisomerase IV. Both of these enzymes are responsible for topological changes in the bacterial DNA during various events of replication and cell division which includes induction of negative supercoils before the initiation of replication, removal of positive superhelical twists in

front of replication fork during elongation, unknotting and compacting the DNA during termination of replication and finally decatenation of interlocked pair of daughter DNA molecule before the separation of the progeny (***Figure 3***) [3]. However, DNA gyrase is only responsible for inducing negative supercoils and different from topoisomerase IV due to its ability to drive the supercoiling in both direction by using ATP cleavage to ADP and P_i as the thermodynamic driving force [4].

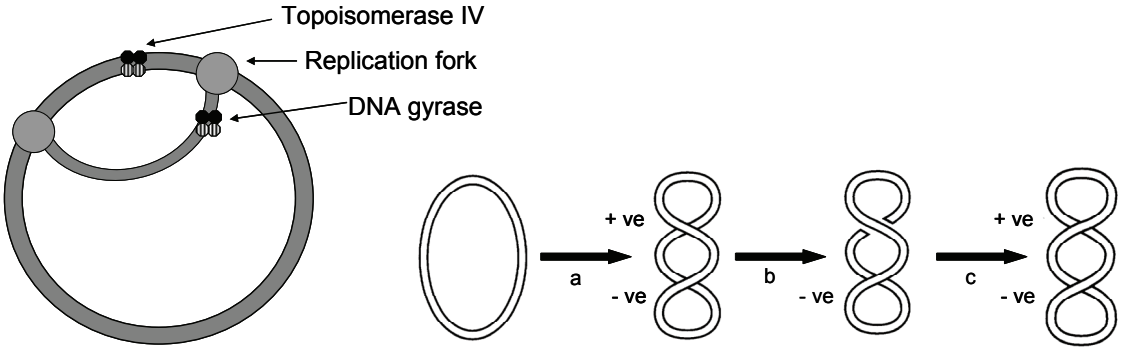

Figure 3. **i)** *DNA gyrase and topoisomerase IV in action* **ii)** *Formation of negative supercoil by DNA gyrase by a) stabilizing positive node b)breakage of the backside strand c) resealing the back side strand in front*

The antibacterial activity of the quinolones is primarily due to inhibition of DNA gyrase. In the presence of quinolones the supercoiling reaction is arrested at the point where the cut ends of the DNA strands are covalently linked to the hydroxyl groups of the tyrosine-122 residues of GyrA. As a result the re-ligation of the broken strands is blocked and the supercoiling reaction has been frozen midway followed by the accumulation of double-stranded nicks in the bacterial genome which in turn halts the essential movements of DNA and RNA polymerases along the DNA template [4] (***Figure 4***). The possible outcome of this condition is recruitment of DNA repair machinery attempts to come to rescue and fails as the recalcitrant quinolone stabilized gyrase-DNA complex persists. All this can evoke the signalling cascade leading to the rapid killing of the bacteria. Usually DNA gyrase in Gram-negative bacteria and topoisomerase IV in Gram-positive bacteria are more sensitive to quinolones, albeit both enzymes are sensitive to some newer quinolones [5].

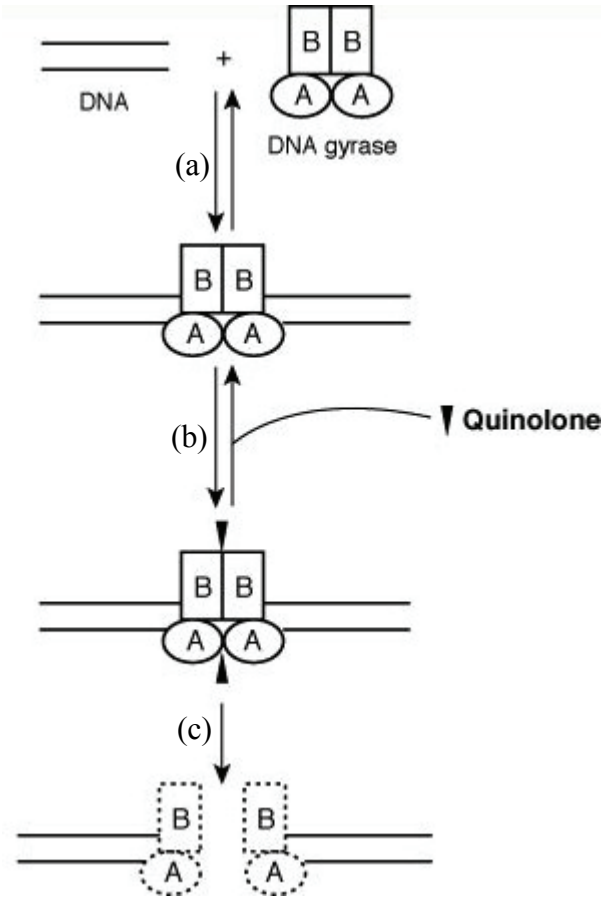

Figure 4. *Intracellular actions of quinolones; a) DNA & DNA gyrase interact, formed a cleaved complex where DNA strands are broken but held by DNA gyrase, b) quinolone stabilizes DNA-DNA gyrase complex, broken strands can not be released and DNA replication is blocked and finally c) broken strands are released and cell death results*

1.2.2 Therapeutic effects of quinolones

The fluoroquinolones are broad spectrum antibiotics and exert their effect in a concentration dependant manner and works effectively at 1 to 4 times concentration of the MIC [6]. Except norfloxacin the other quinolones are well absorbed by gastrointestinal tract and they penetrate well to the lungs, kidney, bones and intestinal walls. Their activity against Gram-negative bacteria is well established including *Escherichia coli*, *Salmonella*, *Shigella*, *Enterobacter* and *Neisseria* species (MIC_{90} < 1 µg/ml). Ciprofloxacin is still the FQ with highest activity against *P. aeruginosa*. In spite of that, it is not recommended now a days as single treatment, since the organism easily becomes resistant during therapy. The third and fourth generation quinolones i.e. trovafloxacin, pazufloxacin, gemifloxacin, and moxifloxacin have improved activity and potency against Gram-positive bacteria *Staphylococcus aureus* and *Streptococcus pneumoniae*. These quinolones have also increased activity against anaerobic bacteria.

1.3 AMINOGLYCOSIDES

The aminoglycosides include an important group of natural and/or semisynthetic highly potent and broad spectrum antibiotics. Since Selman Waksman's discovery of streptomycin from *Streptomyces grisues* in 1944, aminoglycosides have remained an important choice for the treatment of life threatening infections. The discovery of streptomycin was noteworthy because it was the first effective therapeutic for

tuberculosis and the first antibiotic to be isolated from bacterial source. The naturally occurring aminoglycosides are produced by various species of Streptomyces and Micromonospora. The aminoglycosides derived from Micromonospora genera such as sisomicin are expressed by 'micin' instead of 'mycin' e.g. kanamycin from those derived from Streptomyces.

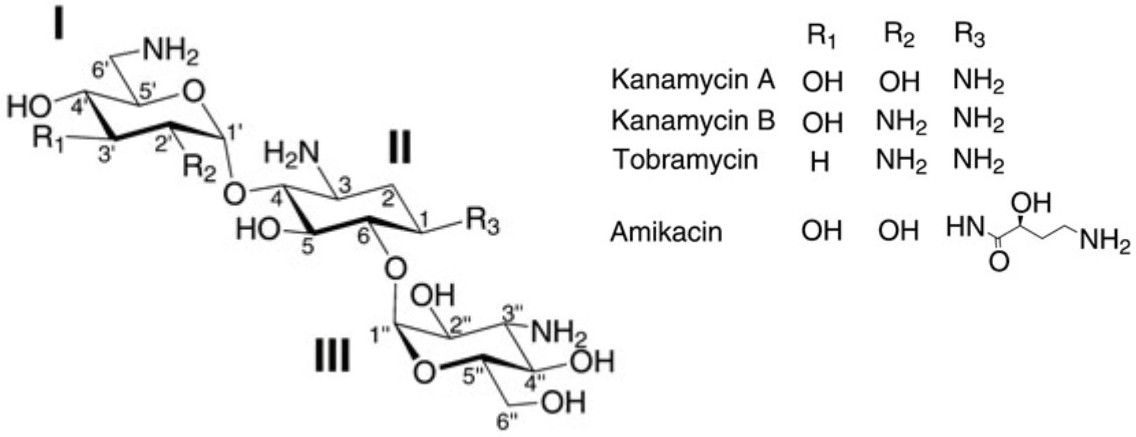

Figure 5. *Deoxystreptamine ring and its derivatives; kanamycin A & B , tobramycin and amikacin*

Followed by streptomycin in the next two decades a number of other naturally occurring compounds including neomycin, kanamycin, tobramycin, gentamicin, sisomicin and paromomycin came into therapeutic use. Among the semisynthetic aminoglycosides amikacin, netlimicin, arbekacin and isepamicin were synthesized from naturally occurring kanamycin, sisomicin, gentamicin and dibekacin respectively. The development of aminoglycoside class was triggered by the necessity to reduce potential drug related toxicity and to avoid development of antimicrobial resistance. Extensive research in the last decades leads to reveal the pharmacodynamic properties of many of the compounds which not only increased their antibacterial activity but also greatly improved their toxicodynamic properties.

Aminoglycosides are characterized by the presence of an aminocyclitol ring linked to aminosugars in their structure. The aminocyclitol ring usually consists of either streptidine or deoxystreptamine; both are derivatives of streptamine. Both neomycin and kanamycin group of aminoglycosides are derived from deoxystreptamine aminocyclitols (***Figure 5***).

1.3.1 Mode of actions of aminoglycosides

The major target of aminoglycoside class of antibiotics is the bacterial ribosome. The intact bacterial ribosome is a 70S particle consisting of a 50S and a 30S subunit that are assembled from three species of rRNA (5S, 16S and 23S) and from 52 ribosomal proteins. Both *in vitro* and *in vivo* experiments with the addition of aminoglycosides resulted in significant decrease in protein synthesis by repression of initiation or

elongation. The 30S smaller subunit of ribosome plays a crucial role in providing high-fidelity translation of genetic material. It contains 16S rRNA which has been found to be the primary target for aminoglycoside induced by prokaryotic ribosomes [7].

The recent advancement in the structural study revealed the molecular interaction between bacterial ribosome and aminoglycosides. A typical bacterial ribosome contains three functionally important tRNA binding sites designated A (aminoacyl), P (peptidyl) and E (exit) sites [8]. High-fidelity (1 wrong amino acid per 3000 polypeptide bonds) of translation is achieved by the ability to differentiate between conformational changes in the ribosome induced by binding of correct or incorrect tRNA at the A site [9]. The 2-deoxystretamine ring containing aminoglycosides increase the error rate of ribosome by incorporating incorrect tRNAs.

The structure of the 30S subunit indicates that two universally conserved adenine residues (A1492 and A1493) are stacked in the interior of helix 44 directly involved in the decoding process during normal translation [10]. When a tRNA binds to the A site results in A1493 and A1492 flip out from their stacked position and G530 also flips out from the *syn* to the *anti* conformation in an energy requiring process. These conformational changes allow the N1 of adenines to interact with the 2′-OH groups of the tRNA residues that are in the first and second positions of the codon-anticodon triplet. Because the distance between the 2′-OH groups will depend on the geometry of base pairing, allowing such hydrogen bonding to discriminate between correct and incorrect tRNAs [11]. The 2-deoxystreptamine ring bind in the major groove of helix H44 of 16S RNA results in the flipping out of A1492 and a 1493 from their stacked position and causes G530 form syn to the anti conformation which normally happens upon binding of tRNAs. These flipping out of conserved nucleotides due to the binding of aminoglycosides to the A site reduce the energy cost allowing the binding of incorrect tRNA to the A site and subsequent mistranslation.

The streptidine ring containing aminoglycosides such as streptomycin also induces the misreading of the genetic code but it acts on ribosome in a different way than 2-deoxystretamine. Mutational demonstration revealed that conformation of bacterial ribosome can have two different conformational states denominated as hyper accurate (increased fidelity of translation) and ribosomal ambiguity (low fidelity or error prone). Binding of streptomycin favours and stabilizes the ribosomal ambiguity state. The non aminoglycoside aminocyclitol spectinomycin inhibits the translocation of peptidyl-tRNA form the A site to the P site and has only bacteriostatic effect [11]. These findings indicate that the misreading of the genetic code is at least partly responsible for the bactericidal effect of characteristic aminoglycosides [7].

Aminoglycoside must cross the outer membrane (in Gram-negative organisms) and the cytoplasmic membrane (in Gram-negative and Gram-positive organisms).The highly positively charged aminoglycosides forms ionic bonding to negatively charged moieties of phospholipids, LPS and outer membrane proteins in Gram-negative bacteria and to phospholipids and teichoic acids in Gram-positive bacteria on the outer membrane surface. This results in self promoted uptake of aminoglycosides across the outer membrane and periplasmic space of Gram-negative bacteria or the cell-wall assembly

of Gram-positive bacteria [12, 13]. Binding of aminoglycosides rapidly displaces the divalent cations from the outer membrane surface which links the adjacent LPS molecules; a process that damages the outer membrane and increases its permeability [7]. The uptake of aminoglycoside through the cytoplasmic membrane is an energy dependent process. In the beginning a very small amount of aminoglycoside gains entry into the cell through an energy requiring process which depends on the proton motive force generated by electron transport system adjacent to the cytoplasmic membrane. The binding of incoming antibiotic to the ribosome results in misreading of mRNA and misfolded proteins and some of the proteins incorporate in the cytoplasm which in turn allows the increase of intracellular antibiotic concentration and rapid killing of the microorganism [12]. Thus microorganisms deficient in electron transport system i.e. anaerobes are intrinsically resistant to aminoglycosides and due to the same reason enterococci and other facultative anaerobes are resistant to low concentration of aminoglycosides.

1.3.2 Therapeutic effects of aminoglycosides

Aminoglycoside antibiotics are poorly absorbed after oral administration due to their hydrophilicity and poor membrane permeability. For the same reason these agents poorly penetrate through intact skins. To achieve rapid and reliable attainment of sufficient peak concentration intravenous administration is generally preferred. To be effective, an antimicrobial must reach and maintain adequate concentrations at the target site and interact with the target site for a period of time so as to interrupt the normal functions of the cell *in vivo*.

Being highly potent and broad spectrum antibiotics the *in vitro* activity of aminoglycosides is notably significant against various Gram-negative pathogens including *Pseudomonas aeruginosa*, *Escherichia coli*, Acinetobacter spp., Citrobacter spp., Enterobacter spp., Klebsiella spp., Serratia spp., Proteus spp. and Morganella spp. The aminoglycosides are also active against *Haemophilus influenzae*, *Neisseria gonorrhoeae*, Salmonella spp., Shigella spp. However this class of agents is not recommended for infections caused by these species as effective and less toxic drugs are widely available. The activity of aminoglycosides against Gram-positive organisms i.e. *Staphylococci* is generally well established but are not generally advocated as single treatment. In most cases an aminoglycoside is frequently administrated in combination with a cell wall active agent (penicillin or vancomycin) to allow synergy in the treatment of serious infections due to streptococci or enterococci. The microbiological spectrum of different aminoglycosides has been shown in ***Table 2***.

1.3.3 Toxicity

The expanded therapeutic use of the aminoglycosides is limited because of toxicity, which varies in form and intensity with the different types of molecules; the main toxic responses are ototoxicity and renal toxicity [14]. Streptomycin and other aminoglycosides target sensory hair cells of the inner ear and can lead to hair-cell degeneration and permanent loss; this leads to irreparable hearing loss in up to 5% of

patients on extended treatment with aminoglycosides [15]. The largely random analyses of structure–activity relationships between the inhibitory and toxicity responses of the aminoglycosides have provided the impressions that the two responses are so closely related in structure–activity terms, a less toxic, equipotent aminoglycoside is unattainable. The development of semi-synthetic aminoglycosides has been largely driven by the goal of finding compounds active against evolving resistant or recalcitrant bacterial pathogens at the same time less toxic. A variety of dosing regimens have also been employed and shown to reduce the incidence of toxicity [16].

Table 2. *Year of deployment and microbiological spectrum of different aminoglycosides*

Aminoglycosides	Year	Microorganisms			
		Staphylococci	Streptococci	Enterobacteria	Pseudomonas
Streptomycin	1944	+++	+	+++	+
Neomycin	1949	+++	+	+++	+
Kanamycin	1957	+++	+	+++	+
Gentamicin	1963	+++	++	+++	+++
Tobramycin	1968	+++	+	+++	+++
Amikacin	1972	+++	+	+++	+++
Netlimicin	1975	+++	+	+++	+++

1.4 CARBAPENEMS

The carbapenems are the ß-lactam antibiotics with broadest spectrum of activity among the ß -lactam class and they are bactericidal against both Gram-positive and Gram-negative organisms. Thienamycin was the first carbapenem discovered in 1970. The unstable nature of thienamycin later led to the synthesis of imipenem [17] and later meropenem which are the widely used carbapenems available in the market (***Figure 6***).

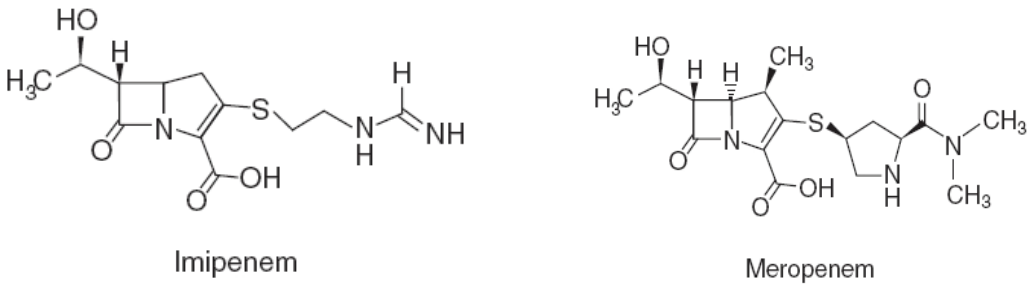

Figure 6. *Chemical structure of imipenem and meropenem*

The carbapenems are differed from other ß-lactam antibiotics as they lack sulphur or oxygen atom in the thiazolinidic ring. Imipenem is subjected to rapid degradation *in vivo* by the enzyme dehydropeptidase-1 (DHP-1) located in the proximal renal tubules of mammals [17] and co administrated with DHP-1 inhibitor (cilastin). Meropenem and other newer penems i.e. ertapenem, doripenem are not subjected to this degradation by DHP-1 due to the presence of 1-ß methyl constituent on the carbapenem nucleus [18]. On the other hand lacking of this side chain makes imipenem not to be a substrate of multidrug efflux pumps in *P. aeruginosa* [19].

1.4.1 Mode of actions of carbapenems

All ß-lactams including carbapenems bind to penicillin-binding proteins (PBPs) and prevent bacteria to complete the cross-linking of peptidoglycan strands. This lack of transpeptidation prevents the synthesis of intact bacterial cell wall. Different carbapenems have varying affinities to PBPs. Imipenem binds preferably to PBP2 followed by PBP1a and 1b and shows weak affinity for PBP3. Meropenem also binds most strongly to PBP2 than to PBP3, also shows strong affinity for PBP1a and PBP1b [17, 20]. In Gram negative bacteria the preferential PBPs for carbapenems are PBP2, PBP1a and PBP3 rather than PBP3 [21]. In *P. aeruginosa* PBP2 is the main target for carbapenems [22]. The uptake of carbapenems is promoted by porin protein OprD.

1.4.2 Therapeutic effects of carbapenems

The carbapenems show a broad spectrum *in vitro* activity against Gram-positive and Gram-negative aerobic and anaerobic bacteria. Imipenem is more potent against Gram-positive bacteria and slightly less potent than meropenem against Gram-negatives [19]. Carbapenems do not show clinically relevant useful activity against *Enterococcus faecium* and *Stenotrophomonas maltophilia*.

1.5 *Neisseria gonorrhoeae*

Neisseria gonorrhoeae belongs to the genus Neisseria, a group of closely related Gram -negative diplocci. DNA-DNA hybridization techniques, numerous taxonomical and sequencing based analyses divided Neisseria into two subgroups (***Table 3***). Further, rRNA and DNA-DNA hybridization analysis have established a subgroup of four species (shown in bold letters ***Table 3***) based on their interspecies relatedness. However they show very different pathogenic profile [23].

Table 3. *Subgroups of Neisseria spp.*

Neisseria	
Subgroup 1	**Subgroup 2**
N. gonorrhoeae, ***N. meningitidis***, *N. subflava,* ***N. lactamica***	***N. cinerea***, *N. polysaccharea*, *N. canis*, *N. denitrificans*, *N. elongata*, *N. macacae*, *N. animalis*, *N. dentiae* and *N. weaveri*

N. lactamica and *N. cinera* are typical nonpathogenic bacteria while *Neisseria gonorrhoeae* causes gonorrhoea in human and *Neisseria meningitides* usually colonizes as commensal to the upper respiratory tract in human but occasionally invades to cause systemic infection and meningitis.

N. gonorrhoeae is oxidase positive Gram-negative coccus, usually seen in pairs with the adjacent sides flattened followed by Gram staining under microscope. *N. gonorrhoeae* is aerobic but can be isolated from parts of human body typical for anaerobic bacteria. In such anaerobic condition it uses nitrate as terminal electron acceptor for anaerobic respiration [24]. It requires CO_2 tension (5% CO_2) and grows optimally at 35^0 to 37^0 C in humid atmosphere and strictly requires glucose as energy source. It is an obligate human pathogen with no other natural hosts. *Neisseria* contains a typical Gram-negative cell envelop which is composed of a cytoplasmic membrane, a thin peptidoglycan layer and an outer membrane. Both *N. gonorrhoeae* and *N. meningitides* share a significant number of major cell wall antigens except the capsule which is never expressed in *N. gonorrhoeae*, however if expressed in *N. meningitides* increases its survival rate in the blood [25].

The specific ability of *N. gonorrhoeae* to adapt, avoid and/or by pass mucosal immune responses and cause repeated infection is achieved by its ability to exhibit both phase and antigenic variation causing each new infection to appear novel. In phase variation the control of expression is on or off whereas in antigenic variation the primary sequence is changed which results in the expression of a different or new epitope in the same protein. The *N. gonorrhoeae* cell surface proteins i.e. pili, LOS and Opa protein are genetically variable. Antigenic variation in pili occurs by the result of intragenic recombination between silent loci *pilS* and donating sequence to the pilin expression locus (*pilE*). The source of donated DNA can be endogenous or exogenous, released by spontaneous cell lysis. The result of this recombination can lead to either phase variation where the expression of pili is switched off or antigenic variation resulting different epitopes in pilin structure which might favour the binding to another type of host cells [26]. *N. gonorrhoeae* expressing pili are competent compared to unpiliated strains and can be transformed naturally [27]. It has also been shown that naturally occurring virulent clones of *N. gonorrhoeae* are more efficient than laboratory strains with transformation rate up to 1% [28].

The Opa protein family is encoded by 11 genes. In any clinical isolates zero to multiple forms of Opa protein can be expressed. *Opa* gene contains repeats of CTCTT in multiple copies in its 5′ end and encodes the hydrophobic core of the leader peptide. The translational frame of these genes is determined by the number of theses repeats. If the number of repeats is out of the frame the gene is switched off resulting phase variation. On the other hand antigenic variation can occur by homologous recombination as seen in pili [29].

The genomic DNA sequence of gonococcal strain FA1090 has been determined (http://www.genome.ou.edu/gono.html) and is approximately 2.2 megabases. Plasmids in *N. gonorrhoeae* play an important role in virulence and antimicrobial resistance (ß-lactamase encoding plasmids). The most studied gonococcal plasmid is a cryptic plasmid (a plasmid with no measurable phenotype) and almost all gonococcal strains harbour this plasmid [23]. In *N. gonorrhoeae* horizontal transfer of genetic material provides an important mean for adaptation and virulence [30]. The DNA is taken up into gonococcal cell from the environment in native double stranded form; although single stranded intermediates can be part of transformation process [23]. DNA fragments containing 10 bp signal sequence GCCGTCTGAA preferentially transformed into gonococcal cells. This sequence are usually found at the coding sequences and repeated 1965 times in the genome of *N. gonorrhoeae* FA1090. The sequence of FA1090 contains pathogenic islands from other bacterial species [31].

1.5.1 Gonorrhoea

Gonorrhoea is a disease restricted to human defined by the presence of *N. gonorrhoeae* in mucosal or other sites. The clinical manifestations of gonorrhoea are urethritis in men and vaginal discharge in women. This organism may also be found in oropharynx, anorectum and in ophthalmic infections in the newborn children and adults. Only approximately 1% of the mucosal infections results in disseminated bloodstream infections [32].

For *N. gonorrhoeae* to create an infection it must be attached to the mucosal surface, followed by entry into the host cell to acquire sufficient nutrients and then evade the host immune response. *N. gonorrhoeae* binds to the host cell receptor CD46 (a member of complement resistance proteins) through its type VI pili which is considered to play the primary role in adhesion and genetic transfer i.e. transformation [33] This prevents GC to be swept away by the cervical secretion in women or urine in men and also diminish the repelling effect of bacterial cell and epithelium due to their negative charge. The secondary attachments are mediated by the opacity associated (Opa) protein which confers a tight attachment by binding to heparin sulphate proteoglycans and CD66 (carcinoembryonic antigens) receptor followed by entry into the epithelial cells [26]. This secondary attachment also aided by lipooligsaccharide (LOS) and gonococcal porin Por. LOS binds to the asialoglycoprotein receptor whereas Por functions as nutrient channel and potentiates the bacterial invasion and transcytosis [33]. After successful adhesion, attachment and invasion, gonococci travel through the epithelial cells by transcytosis and eventually colonize in the subepthelial layer. *N.*

gonorrhoeae needs iron from host cell after colonization. The acquisition of iron by *N. gonorrhoeae* is mediated by transferring-binding proteins which interact with human transferrin and lactoferrin to remove iron directly which is then transported in to the bacterial cell.

Gonococcal infection to the epithelial cells induces the recruitment of cytokines including TNF-α which initiates an inflammatory response or may cause apoptosis. *N. gonorrhoeae* is known to be ingested by macrophages but sialylation of LOS and/or variable expression of Opa proteins have been shown to enhance the ability of the GC to resist the phagocytic killing *in vitro*. The pathogenic *Neisseria* can also secret IgA1 protease which cleaves human IgA1 [23].

Gonorrhoea is mentioned as one of the most common bacterial venereal disease generally spread by sexual activity; however gonococcal eye infection in infants can occur during their passage through the birth canal. In males disease occurs after an incubation period of from 2-14 days characterized by acute urethritis with dysuria and a purulent yellowish green urethral discharge. About 95% of males show symptom of urethritis due gonococcal infection. If untreated may lead to epididymo-orchitis, prostatitis, periurethral abscess or urethral stricture and finally lead to infertility [34]. In females symptoms begins usually 7-21 days after infection. Although a significant portion (>50%) of women is asymptomatic and serve as an important reservoir of *N. gonorrhoeae*. Symptoms include cervicovaginal discharge, bleeding, abdominal or pelvic pain in female. In fewer cases infections spreads from the lower genital tract to ascending upper genital tract causing complicated gonococcal infection defined as pelvic inflammatory disease (PID) in women characterized by salpingitis, endometritis or tubo-ovarian abscesses. Infertility may result form PID and the incidence increases with sequential episodes of infection [34].

1.5.2 Epidemiology

Gonorrhoea remains a common reported communicable sexually transmitted disease worldwide second only to Chlamydia infections. The advent of AIDS and the development of resistance to first line therapies such as penicillin and ciprofloxacin contributed to the epidemiology of gonorrhoea. The incidence of gonorrhoea is largely unknown in developing countries but significantly greater than industrialized countries.

According to WHO in 1999 sixty two million new cases of infection among adults estimated globally. In many industrialized country there was a decline in the number of gonorrhoea cases after the advent of AIDS which has been attributed to changes in sexual behaviour as a result of educational campaign and fear of fatal disease [33]. In Sweden there were 569 cases reported in 2004 and the prevalence is similar to Western Europe [35]. The prevalence was 116/ 100 000 inhabitants in the United States [36]. The highest number of cases was reported in sub-Saharan Africa estimated 17 million followed by 7.5 million in Latin America in 1999. Unlike chlamydial infections gonorrhoea is not evenly distributed amongst the population, with the highest rates in inner cities and in certain subgroups of population such as homosexual or bisexual men,

young people and ethnic minorities, reflecting not only the differences in sexual behaviour but also socioeconomic status and access to health care.

1.6 *Pseudomonas aeruginosa*

P. aeruginosa is an aerobic non-sporeforming Gram-negative rod with remarkable adaptable capacity to survive and persist under a broad range of environmental conditions. It was first isolated in 1872 by Schoroeter from different environmental sources. Taxonomical analysis based on 16S rRNA homology, *P. aeruginosa* belongs to the class of Gammaproteobacteria and the family Pseudomonadaceae. Equipped with large metabolic pathways *P. aeruginosa* can utilize over 80 organic compounds as energy and carbon sources and can grow at temperatures up to 42^0C. Even though classified as an obligate aerobe, it has ability to grow under anaerobic conditions by the presence of an alternative terminal electron acceptor such as nitrite or arginine. This organism is catalase and oxidase positive. The colony morphology of *P. aeruginosa* can be substantially heterogeneous. On a simple agar culture at 37^0C the prototypical colony is large and smooth with an elevated center. Colonies are usually pigmented with blue green colour of copper rust due to the production of blue coloured phenazine pigment pyocyanin (unique to *P. aeruginosa*) and yellow fluorescein [37]. Colony morphology can be altered upon biofilm production, increased antimicrobial and environmental stresses and chronic infections of the human airways [38].

1.6.1 Pathogenecity and epidemiology of *P. aeruginosa*

P. aeruginosa is widely spread in natural environments and typically found in soil and water. It can multiply in distilled water probably by using the gaseous dissolved nutrients. A variety of aqueous solutions including disinfectants, antiseptics, intravenous fluids and eyewash solutions also serve as reservoir of *P. aeruginosa*. It is pervasive throughout the hospital settings and persists in reparatory equipments, sinks, tubs, hydropathy baths etc. Due to their presence in soil they are frequently recovered form fresh vegetables and plants [37]. *P. aeruginosa* is sporadically found as a part of the human microflora of healthy individuals. The organism dies in dry skin of healthy individual and faecal carriages vary between 2% to 10% and probably higher in vegetarians [39].

Although *P. aeruginosa* posses a vast array of virulence factors (***Table 4***) and is ubiquitously distributed in natural environment, this organism is seldom responsible for the community acquired infections in healthy individuals. However, the incidence of *P. aeruginosa* associated infection is high in hospital environment especially in immunocompromised individuals, epithelium compromised CF patients, individual with severe burns, ulcerations, and mechanical abrasions caused by catheterization. *P. aeruginosa* is the major cause of death in CF patients. It is considered to be the leading cause of ventilator-associated pneumonia and urinary tract infections in the intensive care unit [40, 41]. It can also cause infections to soft tissue, bone, joint, ear and cornea.

Emergence of increasing rates of antibiotic resistance *P. aeruginosa* makes treatment of infections mentioned above a serious medical challenge.

1.6.2 Cystic fibrosis (CF)

Cystic fibrosis (CF) is the most common life shortening genetic disease and carried as an autosomal recessive trait about 3% by Caucasians. CF is caused by a defect in the gene involved in the production of a protein known as cystic fibrosis transmembrane conductance regulator (CFTR) that controls the flow of chloride ions into and out of certain cells lining the lungs, pancreas, sweat glands and small intestine. In CF patients defects and/or absence of functional CFTR prevents chloride ion from entering and leaving cells followed by the production of sticky mucus like substance which clogs ducts or tubes in these organs. In the lungs this mucus blocks the airways and results in inefficient clearing of bacteria and occludes phagocytic cells. This favours the persistent colonization and subsequent infection of CF lung by *P. aeruginosa* with a propensity to live in warm and wet environment. *P. aeruginosa* is able to grow in a biofilm in the viscous mucoid respiratory environment in near-anaerobic condition which can trigger the mucoid cell types. The mucoid phenotype can also be triggered by the presence of reactive oxygen species which are often secreted by phagocytic and

Table 4. *Virulence factors* of *P. aeruginosa and their role in infections*

Virulence Factors	Role in Infections
Adhesins	
Type IV pili	• Twitching motility, asialo G_{M1} receptor
Flagella	• Motility and chemotaxis during tissue invasion
Nonpilus mucin-binding adhesion	• Binds to epithelial mucin
Core polysaccharides of LPS	• CFTR protein receptor
Alginate	• Production of biofilm
Type III secretion system (TTSS)	• Secretion and injection of virulence factors into the cytosol of host cells
PcrV	• Translocation of type III secretion system
Xcp secretion system	• Secretion of toxins and enzymes to the extracellular fluid
Toxic proteins	
ExoS	• Affects GTP levels and activities of GTP-binding host cell proteins.
ExoU	• Toxic for macrophages
ExotoxinA	• ADP ribosylates elongation factor 2, stops hosts cell protein synthesis
LasA and LasB	• Acts synergistically to degrade elastin, antibodies and other proteins
Alkaline protease	• Probably plays role to destroy lung surfactant
Phosophlipase C and rhamnolipid	

NK cells present in the CF environment. Living in biofilm is advantageous as it helps the bacteria to escape host immune system and therapeutic concentration of antibiotics. The low or sub-inhibitory concentration of antibiotics reaching the bacteria colonized in a biofilm increase the possibility of developing resistance. The infections are treated and the symptoms subside but it does not eradicate the bacteria completely in chronically colonized patients.

The persistence of *P. aeruginosa* in CF lung involves specific adaptation including adoption of biofilm life style, conversion to mucoidy or small colony variants and loss of virulence gene expression including type IV pili, flagella, exotoxins, LPS, O-antigen and TTSS [42]. This chronic infection may persist for decades ultimately result in loss of lung function and mortality which considered as leading cause of death in CF patients [43].

1.7 RESISTANCE MECHANISMS TO ANTIBIOTICS

Bacterial resistance to antibiotics possesses a major threat to public health concerns. Selection pressure fuelled by the production of large number of antibiotics, widespread use of antibiotics as well as epidemic diffusion of resistant strains are considered as leading cause of bacterial evolution towards resistance.

Intrinsic and acquired resistance

Antibiotic resistance can be intrinsic or acquired. The intrinsic or natural resistance is an inherent capacity of a bacterial species related to its genetic background and does not require any specific target and often involves the presence of low affinity targets, low cell permeability or efflux mechanisms [44, 45]. For example *P. aeruginosa* exhibits low level of resistance to fluoroquinolones or aminoglycosides due to intrinsically expressed efflux pumps, or inactivation of ß-lactam antibiotics due to chromosomal ß-lactamase. Intrinsic resistance is considered as normal behaviour of the species in the presence of antibiotics. Knowledge about intrinsic resistance mechanisms is important to predict potential emergence of antibiotic resistance under selective pressure.

Whereas in acquired resistance a bacterial species which is normally sensitive to a specific class of antibiotic become resistant as a result of 1) changes in the bacterial chromosome due to selection pressure and 2) acquisition of resistance determinants from the chromosome of other species and mobile elements such as plasmids or transposons. Alteration of antibiotic target with decreased affinity due to point mutations in GyrA subunit of *N. gonorrhoeae*, *P. aeruginosa*, *E. coli* or *S. aureus* is one of the important examples of acquired resistance mechanism. Increased efflux of antibiotics due to regulatory gene mutation or modifications of antibiotics by plasmid borne aminoglycoside modifying enzymes are important examples.

The distinction between intrinsic and acquired resistance is of great importance because the later has great risk to be transferred to a sensitive, intrinsically resistant strain or to another species to give rise to new resistant mechanism.

The mechanisms of acquired resistance are multiple and sometimes overlap with intrinsic resistance. For each class of antibiotics there is at least one mechanism that allows specific bacterial species to protect itself against the action of antibiotic and can be divided into five main categories (***Table 5***)

Table 5. *Mechanisms of antimicrobial resistance*

Resistance mechanisms	Results	Example
Alteration of antibiotic targets	Antibiotic is no longer capable of reacting or binding with the targets and exerts its effect	• FQ resistance in *N. gonorrhoeae*
Inactivation of antibiotics		• Enzymatic inactivation of aminoglycosides by AMEs
Defects in antibiotic penetration	Loss of porin proteins	• Carbapenem resistance in *P. aeruginosa* due to downregulation of OprD
Extrusion of antibiotics by efflux	Increased expression of efflux pumps	• MexXY efflux mediated aminoglycoside resistance in *P. aeruginosa*
Protection of the targets	Antibiotic is unable to interact with its target	• *qnr* protects DNA gyrase from the action of quinolone in *K. pneumoniae*

1.8 MECHANISMS OF RESISTANCE TO FLUOROQUINOLONES

1.8.1 Alterations in target enzymes

The most important mechanism of fluoroquinolone resistance is chromosomal mutation in the genes encoding the subunits of DNA gyrase and topoisomerase IV or both. In a bacterial population these alterations exist in small numbers ($1/10^6$ to $1/10^9$). The amino acid changes in GyrA subunit of DNA gyrase regardless of bacterial species are generally localized around the active site where the Tyr122 is covalently linked broken DNA strand during enzyme action [46]. The spanning amino acids 67 to 106 within this locus is called the quinolone-resistance-determining-region (QRDR), as shown in *E. coli* and the most important positions are Ser83 and Asp87. In case of ParC subunit the QRDR region spans between amino acids 61 to 122 and the corresponding hotspot positions include Ser79 and Asp83. Mutations in GyrB and ParE subunit are much less common than those in GyrA and ParC and usually localized to the mid portion of the subunits in a domain involved in interactions with their complementary subunit. The initial step in mutational resistance is achieved by an amino acid change in the most

sensitive enzyme generally DNA gyrase in Gram-negative and topoisomerase IV in Gram-positive. Higher levels of resistance occur by sequential addition of mutation in both subunits. For example, the commonest substitution in *E. coli* is Ser83 to either Leu, followed by Ser83Val or Ser83Ala and results in 40 fold increase in MIC. However double mutation at Ser83 and Asp87 confers higher levels of MIC [3, 5, 47].

1.8.2 Efflux mediated fluoroquinolone resistance

Resistance to fluoroquinolones can also result from the decreased accumulation of the drug inside the bacterial cell due to increased efflux. The efflux determinants of fluoroquinolone resistance are multidrug transporters encoded by endogenous chromosomal genes. However, it is mostly members of a single resistance/nodulation/division super family (RND) found in Gram-negative species that are implicated in clinically relevant resistance. A typical RND transporter is composed of at least 3 components: an inner membrane spanning pump protein pump protein that works in conjunction with a periplasmic membrane fusion protein and an outer membrane protein which favours the efflux of favourable substrate both from inside the cell and periplasmic space [48, 49]. Many transporters have been demonstrated to be essential for cellular invasion and resistance to natural host substances, such as bile salts and specialized host-defence molecules [50]. Efflux mediated fluoroquinolone resistance was found to play a significant role in *E. coli* and *P. aeruginosa* and also described in *S. aureus* and many other clinically relevant bacteria. In *E. coli* the multiple antibiotic resistance (*mar*) locus is responsible for resistance to fluoroquinolones and other structurally unrelated antibiotics [51].

In *P. aeruginosa* at least 4 RND type multidrug efflux systems are involved in quinolone resistance. Two of them MexAB-OprM and MexXY-OprM are constitutively expressed providing baseline or intrinsic resistance to fluoroquinolone antibiotics. MexCD-OprJ and MexEF-OprN efflux systems are involved in acquired quinolone resistance in *P. aeruginosa*. Exposure of *P. aeruginosa in vitro* to 12 different quinolone antibiotics showed that the predominant resistance mechanism was selected for efflux type mutant. Newer quinolones favoured the MexCD-OprJ system whereas older quinolones are selected for MexEF-OprN and MexAB-OprM [52]. In another study conflicting results have been reported regarding which resistance mechanism is preferentially selected by *P. aeruginosa* in response to quinolone exposure. At concentrations close to the MIC, efflux-type mechanisms were selected almost exclusively in the laboratory strain PAO1. The gyrase type mutations appeared only at quinolone concentrations above 4x MIC. In *N. gonorrhoeae* there is no direct role of efflux pump in quinolone resistance but Dewi *et al.* reported that combination of mutations in the QRDR and the regulatory region of MtrAB suggest a role of efflux mediated elevated quinolone resistance in *N. gonorrhoeae* [53].

1.8.3 Plasmid mediated fluoroquinolone resistance

Plasmid mediated quinolone resistance has been reported by Martinez *et al.* A multi resistance plasmid pMG252 from clinical isolate of *Klebsiella pneumoniae* harbours a

gene known as *qnr* which protects DNA gyrase from the action of quinolone [54]. Interspecies transfer of this plasmid to *Enterobacteriaceae* and *P. aeruginosa* also conferred resistance to quinolone [55]. However, the presence of such transferrable type of quinolone resistance in clinical isolates seems to be very rare and did not require too much attention probably due to the fact that quinolones themselves can eliminate plasmid [56, 57].

Table 6. *Summary of quinolone resistance mechanism*

Organism	Primary target	Secondary target	Efflux
E. coli	GyrA	GyrB, ParC, ParE	AcrAB
P. aeruginosa	GyrA	ParC	MexAB-OprM
			MexCD-OprJ
			MexEF-OprN
			MexXY-OprM
N. gonorrhoeae	GyrA	ParC	MtrAB?

1.8.4 Fluoroquinolone resistance in *N. gonorrhoeae*

Fluoroquinolone resistance in *N. gonorrhoeae* is mainly attributed to point mutations in the QRDR of the bacterial gene *gyrA* and *parC* [58]. It has been described that the alterations in position Ser91 and Asp 95 in *N. gonorrhoeae* correspond to the *E. coli* QRDR hotspot position Ser83 and Asp87 [59]. Mutations in the QRDR of *N. gonorrhoeae gyrA* have been found in strains susceptible to ciprofloxacin (MIC < 1 mg/L) and mutations in *parC* QRDR do not alone confer resistance to ciprofloxacin, however presence of double mutation in *gyrA* or combination of *gyrA* and *parC* QRDR mutation mostly contribute to ciprofloxacin resistance (MIC $\geq$ 1 mg/ L) [58, 60-62].

The most common GyrA and ParC QRDR alterations contributing to higher MICs ($\geq$ 1 mg/L) to quinolone in resistant *N. gonorrhoeae* includes Ser91 to Phe [58, 63] or Tyr [64] and Asp95 to Gly, Asn [58, 59, 63, 64], Ala [65], Tyr [66, 67] and His [68]. Alterations in other positions have been reported by many groups. For example substitution of alanine to serine in position 67 and 75 as well as to proline in position 85 in three different *N. gonorrhoeae* strains all harbouring wild type ParC resulted in increase in ciprofloxacin MIC from 0.004 to 0.63 mg/L. But the effect of these changes was not correlated to MICs as the alterations at position 91 and 95 were also included [69]. A combination of Ser91Ile and Ser87Arg gave MIC of ciprofloxacin 0.25 mg/L was also reported [68].

The main ParC QRDR mutations contributing to ciprofloxacin resistance ($\geq$1 mg/L) includes substations at positions Asp86, Ser87, Glu91 and Ala92. Other important positions include Arg116, Gly85 [70].

It has been showed that introduction of mutation by transformation at GyrA position 91 and 95 resulted in increase MIC of ciprofloxacin of 0.25 mg/L [58]. As GyrA is the primary target for quinolone resistance, introduction of a single point mutation at position 91 increased the MIC of ciprofloxacin form 0.03 to 0.13 mg/L whereas double mutation in GyrA with wild type ParC appear to increase the MIC of ciprofloxacin ≥ 0.13 mg/L [65].

1.8.5 Antibiotic uptake in *N. gonorrhoeae*

A reduced quinolone uptake-related resistance mechanism has also been reported from some studies. As reported by Tanaka *et al.* reduced uptake and accumulation can be a mean of resistance to norfloxacin in clinical isolates from Japan [71] even though this group did not report alteration in any specific porin protein. Later on, two other groups reported the relationship between ciprofloxacin susceptibility and alteration in gonococcal porin protein PorB1 where alteration of two amino acids Gly120 to Asn and Ala21 to Asp in loop 3 as well as changes in loop 5 has been accounted for resistance to ciprofloxacin [72, 73]. However, no correlation was found between the above mentioned changes and ciprofloxacin resistance by Veresshchagin *et al.*[74].

1.9 MECHANISMS OF RESISTANCE TO CARBAPENEMS

Carbapenems are stable against almost all ß-lactamases including AmpC ß -lactamases and extended-spectrum-ß-lactamases (ESBLs) which made them suitable choice of antibiotics to treat Gram-negative bacteria that are already resistant to other ß -lactams including third generation cephalosporins [17]. Despite of their broad spectrum of activity some organisms demonstrate intrinsic resistance to carbapenems. Representative examples are the poor binding affinity of all ß-lactams including carbapenems to PBP2a in MRSA and PBP5 in *E. faecium* account for the resistance in this organisms [17, 20]. Moreover increased production of PBP5 with decreased affinity to imipenem has also been observed in moderate imipenem resistant ampicillin sensitive *E. faecium* [75]. Also the presences of specific acquired carbapenem-hydrolyzing β-lactamases can lead to the rapid hydrolysis of carbapenems. The presence of carbapenemases has been confirmed in *P. aeruginosa*, *Serratia marcescens, A. baumannii* and other Gram-negative organisms. In *P. aeruginosa* downregulation of porin protein OprD and overexpression of efflux pump MexAB-OprM) also confer resistance to carbapenems. In the next section carbapenem resistance mechanisms in *P. aeruginosa* will be discussed.

Carbapenems resistance mechanisms in *P. aeruginosa*

The known carbapenem resistance mechanisms in *P. aeruginosa* can be attributed by control of intracellular concentration of the antibiotic via decreased production of outer membrane porin OprD [76] or increased efflux from inside to the outside of cell [77]

and hydrolysis by metallo ß-lactamases [78, 79]. Other mechanism may involved is decreased affinity or expression of penicillin binding proteins [80].

1.9.1 Outer-membrane proteins

The outer membrane of *P. aeruginosa* functions as a protective permeability barrier that slows down the penetration of antibiotics and other noxious compounds into the cell. However, many substrates necessary for the growth of the cell must pass through this permeability barrier and in *P. aeruginosa* this is achieved by the presence of water-filled protein channels known as porin located in the outer membrane. *P. aeruginosa* has 3 large families of porins; the OprD family of specific porins, the OprM families of efflux porins and the TonB family of gated porins [81]. These porin proteins are involved in the bacterial cell functions of iron-siderophore, glucose, amino acid, and phosphate transport [82]. However, these porin channels also serve as the passage for hydrophilic antibiotics such as ß-lactams, aminoglycosides, tetracyclines and some fluoroquinolones [83, 84].

The outer membrane protein OprD (443 amino acids) is a basic amino acid transporter and loss of this function causes porin-associated resistance to carbapenem antibiotics [85]. The chemical resemblance between carbapenem and basic amino acids allow its binding to the external loop 2 (amino acid positions 96-134) and 3 (amino acid positions 157-203) [86] and mutations in these regions confer resistance to carbapenems [86]. However amino acid alterations in external loop 5 (amino acid positions 260-274), 7 (amino acid position 353-396) and 8 (amino acid position 421-434) result in expansion of the channel and lead to hyper-susceptible phenotype [81, 87]. The most important OprD associated carbapenem resistance is loss or down-regulation of OprD [76, 88, 89]. In clinical isolates down regulation of OprD causes imipenem MIC to increase from 1-2 mg/L to 8-32 mg /L and meropenem MIC only to 2-4 mg/L. The mechanism by which OprD is decreased is diverse. Reports from some investigators showed that mutations within the structural gene *oprD* or the putative promoter region can account for loss of OprD [85].

In addition to mutations specifically associated with *oprD* and its promoter, down-regulation of OprD in mutant *P. aeruginosa* is also associated with the overexpression of MexEF-OprN. These mutants exhibit increased expression of the *mexEF-oprN* through the action of a transcriptional regulator MexT. Although a consensus binding region has not been identified upstream of *oprD*, expression of cloned MexT from a plasmid has been shown to repress the expression of OprD which was sufficient to significantly increase MIC of imipenem [90, 91]. MexS (PA2491) and *mvaT* (PA4315) has shown to have similar effects [92]. OprD is also repressed by salicylates and catabolic expression and activated by arginine and some other amino acids [81].

1.9.2 Efflux mediated carbapenems resistance

In *P. aeruginosa* 3 efflux pumps have been shown to play relevant role in carbapenem resistance; MexAB-OprM, MexCD-OprJ and MexXY-OprM [93]. MexEF-OprN has been found to be inversely co-regulated with OprD through the common regulator MexT but carbapenem is not a substrate for this efflux pump [52, 94]. Meropenem,

doripenem and ertapenem are all substrates for efflux pumps whereas imipenem is not due to the lack of heterocyclic side chain [19]. MexAB-OprM is regarded as the most effective in pumping out carbapenems and a modest 2-fold increased expression can achieve clinically relevant MICs whereas increases in transcriptional level by 10 and 70 fold for MexXY and MexCD-OprJ is required respectively to confer the same degree of resistance [88, 95]. A combination porin protein OprD down regulation and increased transcription of MexAB-OprM system can increase the meropenem MIC from wild-type up to 16 mg/L [96].

1.9.3 Carbapenemases

As mentioned earlier carbapenems are stable against almost all ß-lactamases including AmpC-ß-lactamases and extended-spectrum- ß –lactamases (ESBLs). However reports of carbapenem-hydrolyzing β-lactamases also known as carbapenemases have been increasing over the last few years. Carbapenemases are comprised of a rather heterogeneous mixture of β-lactamases belonging to either molecular Class A, which has a serine in the active site of the enzyme, or molecular Class B, the metallo- β-lactamases which have zinc in the active site [97].

The class A enzymes hydrolyze imipenem well enough to provide resistance but they show much higher hydrolysis rates for ampicillin and have been found in strains that also possess an AmpC β-lactamase. The class A carbapenemases include chromosomal, plasmid or integron encoded enzymes. Clavulanate can inhibit class A carbapenemases. The most notable example of the plasmid serine carbapenemases are the *Klebsiella pneumoniae* OXA-type carbapenemases [98].

Metallo-β-lactamases confer resistance to carbapenems, cephalosporins and penicillins. They can be chromosomal, plasmid or integron encoded enzymes in diverse genera of Gram-positive and Gram-negative bacteria. Metallo-β-lactamases are inhibited by EDTA, but not by the β-lactamase inhibitors [99, 100] and can all hydrolyze imipenem at a measurable rate however, they differ in their abilities to hydrolyze other β-lactam substrates [101]. Up until now, 4 groups of carbapenemases have been reported in *P. aeruginosa*; IMP (Imipenemase), VIM (Verona Imipenemase), GIM (German Imipenemase) and SPM (Sao Paulo metallo- β-lactamase).

This combination of metallo- and serine-based β-lactamases confers resistance to all classes of β-lactam antibiotics. Although attempts have been made to develop inhibitors that would inactivate the metallo-β-lactamases, there is currently no β-lactamase inhibitor available that can be used against these enzymes [102].

1.9.4 Penicillin-binding proteins

Penicillin-binding proteins (PBPs) are involved in the final stages of the synthesis of peptidoglycan, which is the major component of bacterial cell walls. They are attributed to the name penicillin binding protein because of their affinity for and binding of penicillin. PBPs have been shown to catalyze a number of reactions involved in the process of synthesizing cross-linked peptidoglycan from lipid intermediates and

mediating the removal of D-alanine from the precursor of peptidoglycan. Purified enzymes have been shown to catalyze the following reactions: D-alanine carboxypeptidase, peptidoglycan transpeptidase, and peptidoglycan endopeptidase. In all bacteria that have been studied, enzymes have been shown to catalyze more than one of the above reactions [103]. The enzyme has a penicillin-insensitive transglycosylase N-terminal domain (involved in formation of linear glycan strands) and a penicillin-sensitive transpeptidase C-terminal domain (involved in cross-linking of the peptide subunits) and the serine at the active site is conserved in all members of the PBP family [104].

Table 7. *P. aeruginosa penicillin binding proteins.*

PBPs	Gene names	Locus ID	Location	Type	Corresponding PBP in *E. coli*	References
PBP-1a	*ponA*	PA5054	5681kb	HMW(A)	PBP-1a	[105]
PBP-1b	*ponB*	PA4700	5280kb	HMW(A)	PBP-1b	[106]
PBP-2	*pbpA*	PA4003	4485kb	HMW(B)	PBP-2	[107]
PBP-3	*pbpB*	PA4418	4954kb	HMW	PBP-3	[108]
PBP-3a	*pbpC*	PA2272	2501kb	HMW	PBP-3 homolog	[109]
PBP-4	*dacB*	PA3047	3410kb	LMW	PBP-4	[110]
PBP-5/6	*dacC*	PA3999	4480kb	LMW	PBP-5	[111]
PBP-7	*pbpG*	PA0869	950kb	LMW	PBP-7	[112]

Bacteria encode for multiple PBPs with different roles in cell division and broadly classified into high-molecular-weight (HMW) and low-molecular-weight (LMW) categories [104]. In *P. aeruginosa* 8 different PBPs have been identified up to date; PBP-1a, -1b, -2, -3, -3a, -4, -6/-5 (depending on the nomenclature) and -7 that are homologues of *E. coli* PBPs; -1a, -1b, -2, -3, -4, -5 and -7 [105, 112-114]. The PBPs of primary importance include the essential high molecular weight PBPs 1a, 1b, 2, 3, carbapenems show the greatest affinity for PBP 2 in *Escherichia coli* and *Pseudomonas aeruginosa* [19, 115] (***Table 7***). The inhibition of PBP 2 causes changes in cell morphology leading to the formation of spherical cells, whereas inhibition of PBP 3 leads to filamentation [116, 117].

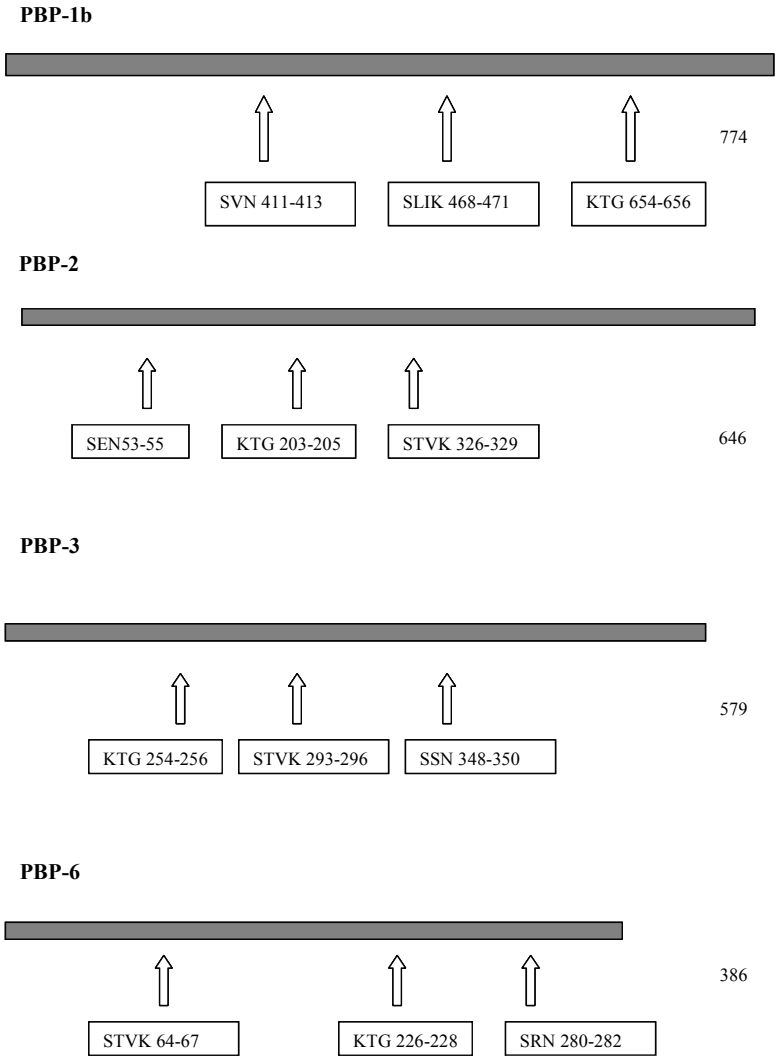

Figure 7. *Schematic view of the conserved regions of major PBPs in P. aeruginosa*

Carbapenems in general have high affinity for multiple PBPs in Gram-negative bacteria [19]. In one report decreased susceptibility to carbapenem was correlated with decreased binding affinity to carbapenem in a clinical *P. aeruginosa* strain [118]. In clinical isolates of *Acinetobactor baumannii* decreased susceptibility to carbapenems has also been correlated with decreased expression of PBP-2. In *E. coli* imipenem and meropenem both have high affinity for PBP-2 but imipenem has lower affinity for PBP-3, unlike meropenem which also has affinity for PBP-3, although to a lesser degree than to PBP 2 [19, 115].

Therefore alterations of PBP-2 and PBP-3 can be speculated as carbapenem resistance mechanism in *P. aeruginosa*. Both downregulation of PBP-2 and alteration of amino acids in the regions close to 3 conserved motifs S-X-N, K-T-G and S-X-X-K (***Figure 7***) [119] in PBP-2 and PBP-3 could be attributed as carbapenem resistance determinants.

1.10 MECHANISMS OF RESISTANCE TO AMINOGLYCOSIDES

Aminoglycosides group of antibiotics are broad-spectrum antibacterial agents with desirable bactericidal activity against difficult-to-treat Gram-negative bacteria and mycobacteria. However the emergence and dissemination of resistant strains have somewhat reduced the potential of these antibiotics in empiric therapies. Emergence of resistance also responsible for the decline in interest in these antibiotics and there have been no new aminoglycoside antibiotics brought to the clinic for over two decades. The key to the successful deployment of the next generation of aminoglycoside antibiotics is evasion of existing resistance mechanisms. Aminoglycoside resistance occurs by three methods: modification of aminoglycoside transport (import and efflux), modification of the rRNA and ribosomal protein targets and via the synthesis of aminoglycoside-modifying enzymes. As the aminoglycoside antibiotics are a vital component of antipseudomonal chemotherapy in the treatment of a variety of infections, particularly pulmonary infections in CF patients, in the next sections aminoglycoside resistance mechanisms will be discussed in *P. aeruginosa.*

Aminoglycosides resistance mechanisms in *P. aeruginosa*

1.10.1 Decreased uptake or increased efflux

Decreased aminoglycoside concentration inside a target cell, by reduction of drug uptake will affect the susceptibility of the strain to the whole class of aminoglycoside compounds and can be the cause of impermeability-type or adaptive resistance.

The impermeability phenotype generally confers low to moderate levels of pan-aminoglycoside resistance and is most frequently encountered in *P. aeruginosa*, particularly among isolates from cystic fibrosis patients [120, 121]. It has been frequently attributed to modifications in LPS structure which are thought to reduce the ability of aminoglycosides to cross the outer membrane [122, 123]. Clinical strains showing low level resistance to gentamicin were shown to have a modified, less negatively charged, lipopolysaccharide that exhibits a lower affinity for gentamicin [124]. In addition the viscous polyanionic extracellular alginate produced by mucoid strains of *P. aeruginosa* was shown to act as a physical and ionic trap to reduce the uptake and early bactericidal effect of aminoglycosides [125].

Since uptake of aminoglycosides is an energy-requiring phenomenon, mutations that affect the membrane potential can confer aminoglycoside resistance [126, 127]. In a report it has been demonstrated that respiratory chain mutants or strains containing functional mutations in their ATP synthases were shown to exhibit decreased susceptibility to aminoglycosides [128, 129]. Such mutants have been isolated from clinical or experimental endocarditis caused by infection with *Escherichia coli*, *S. aureus*, or *P. aeruginosa* [130]. Supplementation of the growth medium with menaquinone (a lipophilic quinone required for electron transport) biosynthesis precursor restored the aminoglycoside sensitivity in a *Bacillus subtilis* aminoglycoside

uptake deficient mutant [131]. Similarly, quinone auxotrophs of *S. aureus* have an aminoglycoside resistance phenotype that can be abolished by the addition of menaquinone precursors to the medium [128]. The small colony variants of various pathogens, such as *S. aureus* or *P. aeruginosa* have reduced rates of aminoglycoside uptake due to changes in cytoplasmic membrane proteins or alterations in energy dependent uptake across the inner membrane or mutations in heme or menaquinone biosynthesis [132, 133].

Adaptive resistance can occur partially as a result of induction of anaerobic respiration genes upon exposure to aminoglycosides [134] and verified by the fact that bacteria grown under anaerobic or low pH conditions exhibit a general aminoglycoside transport defect [135]. Adaptive resistance, where the initial rapid accumulation of drug and killing effect in cell populations is followed by a change to slow accumulation and corresponding reduced susceptibility, which is reversible upon removal of the aminoglycoside, has often been observed *in vitro* and *in vivo* in *P. aeruginosa* and is a consideration in establishing effective dosing regimens [136, 137].

1.10.2 Efflux mediated aminoglycosides resistance

Energy-dependent bacterial efflux is reasoned as one of the major cause of antibiotic resistance. Efflux mediated antibiotic resistance is particularly true for the multidrug-resistant opportunist pathogens responsible for nosocomial infections, which have to counter the environmental pressure exerted by the constant presence of antibiotics. It was first described as a mechanism of resistance to tetracycline in *E. coli* [138, 139]. In next two decades both agent and class specific numerous chromosome and plasmid encoded drug/multidrug efflux transporters have been described in a variety of microorganisms. Based on their amino acid sequence similarity bacterial efflux transporters capable of extruding antimicrobials usually divided into 5 classes; the ATP-binding cassette (ABC) family, the major facilitator superfamily (MFS), the resistance-nodulation and -cell division (RND) superfamily, the small multidrug resistance (SMR) family and the multidrug and toxic compound extrusion (MATE) superfamily [140].

The RND family of efflux transporter are of particularly interesting in Gram-negative organism due to their abundance and contribution to antibiotic resistance [141]. The genes encoding the multidrug efflux systems are almost invariably encoded by chromosomal genes that are expressed constitutively to contribute intrinsic resistance or following mutation to contribute acquired resistance [142]. The contribution of constitutive and active drug efflux work synergistically to manifest resistance [143, 144].

At least 7 multidrug efflux transporters in *P .aeruginosa* have been found to be involved in antibiotic resistance and 5 of them are well characterized ***Figure 8***. These efflux pumps typically operate as a tripartite system that includes the cytoplasmic membrane protein that functions to efflux antibiotics across the cytoplasmic membrane,

an outer membrane channel protein that provides the passage of drugs to the outside of the cell and a linker protein that connects the two pump a membrane fusion protein (MFP).

Although the efflux pumps in *P. aeruginosa* that have been characterized so far share common structural organization but they are far different from each other in many

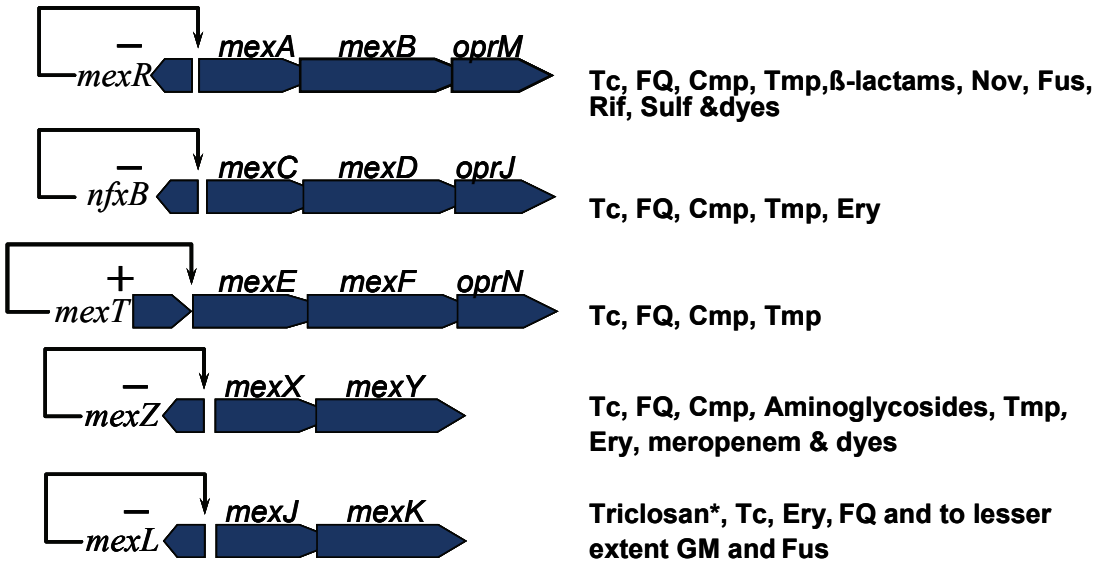

Figure 8. *Schematic view of well characterized P. aeruginosa efflux pump systems.*

respects including the substrate antibiotics they extrude and regulation of their operon. However, only the *mexXY* efflux system has been found to contribute both impermeability and adaptive resistance to aminoglycoside antibiotics in this organism [145-149].

MexXY efflux was found to be accountable for aminoglycoside resistance in *P. aeruginosa* strains which were characterized as aminoglycoside impermeability-type resistance (AGIR), the single most common resistance mechanisms in 90% of strains isolated form CF patients[149]. Deletion of *mexXY* from wild type *P. aeruginosa* increased their susceptibility to gentamicin, tetracycline and erythromycin [145]. The substrate specificity of this efflux system is relatively wide which includes macrolides, tetracyclines, chloramphenicol, novobiocin, fluoroquinolones and ß-lactams [150-152]. MexY is a drug proton antiporter associated with the membrane fusion or linker protein MexX [153] and homologous to AcrD in *E. coli* [154]. MexX and MexY are encoded by the *mexXY* operon and the outer membrane component for this system is apparently OprM the product of the third gene encoding the RND type three-component operon MexAB-OprM [145, 151]. In addition, the *P. aeruginosa* outer membrane proteins OpmG and OpmI appear to be involved in intrinsic resistance to aminoglycosides, potentially as additional outer membrane channel components of the MexXY pump or

another efflux pump [155]. Expression of MexY is strongly induced by agents interfering with protein synthesis such as aminoglycosides or tetracycline [156]. Although inactivation of the pump in resistant strains increased susceptibility confirming a contribution, and in CF aminoglycoside resistance *P. aeruginosa* strains the pump protein is highly expressed but there is not always a clear association between expression level and resistance [147, 157].

The expression of MexXY is negatively regulated by another protein, MexZ, encoded by a gene that is transcribed divergently and located 263 bp upstream of *mexX*. MexZ contains a helix-turn-helix motif at its N-terminal end that is attributed to DNA binding domain [145]. Inactivation of this regulator increases expression of MexXY. However, this did not lead to aminoglycoside resistance [149], and the pump is apparently still further inducible by drugs even when *mexZ* is mutated [156], suggesting that regulation of MexXY expression is not controlled solely by MexZ. This finding is also notion with the fact that aminoglycoside resistant clinical isolates expressing MexXY lacking alterations in MexZ. However in CF clinical isolates of *P. aeruginosa* presence of numerous *mexZ* mutation and high level of MexXY expression indicates a role of mexZ as a negative regulator which may involve in overexpression of *mexXY* in these strains [148, 157].

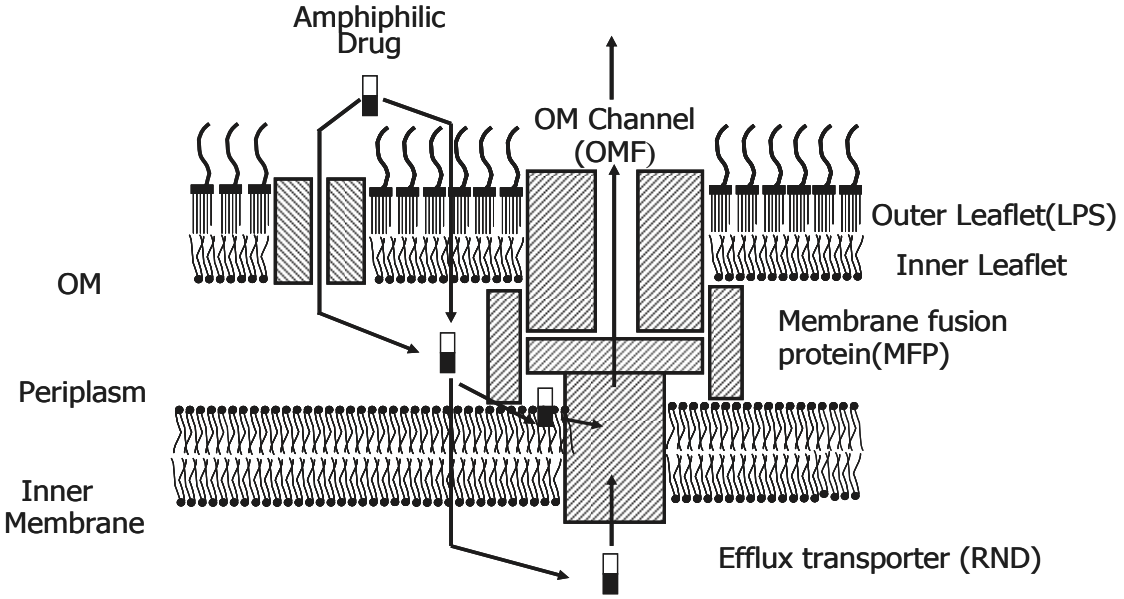

Figure 9. *The efflux transporter (MexY) in P. aeruginosa is connected to the outer membrane (OM) channel proteins (OprM) via the membrane fusion protein (MexX) and thereby forming a channel spanning the inner and outer membranes. The efflux pump MexXY-OprM is capable of extrusion of aminoglycosides from the cytosol as well as from the periplasmic space contribute to both intrinsic and adaptive resistance.*

Moreover, MexZ or other regulatory components apparently do not directly interact with inducing compounds, but rather, the impact of drugs at the ribosome may be generating an intracellular signal(s) leading to the regulatory cascade [156]. Several non-enzymatic mechanisms of gradual increase in aminoglycoside resistance which may directly or indirectly interact with MexZ to facilitate the induction of *mexXY* have also been reported [158].

RND pump components (***Figure 9***) recognize and transport aminoglycosides directly [159], it stands to reason that the MexXY pump could have a natural function in extruding a toxic molecule related to the interference with protein synthesis. The advantage of having the inducible pump expressed constitutively in *P. aeruginosa* is the elimination of lag time for induction of the pump in cell populations intermittently exposed to aminoglycosides, which may be the case particularly in lung infections.

1.10.3 Aminoglycoside modifying enzymes

Aminoglycoside modifying enzymes (AMEs) covalently modify the aminoglycoside antibiotics. Modified aminoglycosides binds poorly to the ribosomal A-site target [160] and fail to trigger the energy dependant phase II allowing the bacteria to survive in the presence of the drug. Modifying enzyme-based resistance to aminoglycosides has been known for decades, consistent with aminoglycosides being natural products derivatives (i.e. protection determinants would exist for producing organisms) [161].

Three categories of modification enzymes have been described in the bacterial cytoplasm; aminoglycoside acetyltransferase (AAC), aminoglycoside phosphoryltransferase (APH) and aminoglycoside nucleotidyltransferase (ANT) which acetylate, phosphorylate and adenylate aminoglycoside antibiotics respectively. These enzymes are further subdivided into classes based on their site of modification of the drug and the spectrum of resistance [162]. For example AACs can acetylate aminoglycosides at the 1, 3, 2′ and 6′ amino groups, and are correspondingly designated AAC(1), AAC(3), AAC(2′) and AAC(6′) respectively. Individual variants of these classes are further subdivided using roman numerals, such as AAC(3)-I, II and III. Aminoglycoside modifying enzymes are in most of the cases mobile, being carried on R factors, transposons and integrons but can also be encoded from chromosome [163]. These elements can therefore transfer readily and are often found on mobile elements with other resistance determinants, providing multidrug resistance to sulphonamides, chloramphenicol, antiseptics and perhaps most worrisome, ß-lactams [164, 165]. AMEs of differing specificities can also accumulate to provide panaminoglycoside resistance [166-168].

Aminoglycoside modifying enzymes are common determinants of aminoglycoside resistance in *P. aeruginosa* [169, 170] and also individual *P. aeruginosa* can carry multiple aminoglycoside modifying enzymes and can show broad spectrum aminoglycoside resistance [167, 168].

Aminoglycoside acetyltransferase (AAC) catalyzes the acetyl-CoA-dependent N-acetylation of one of the four amino groups of typical aminoglycosides (***Figure 10***). They include enzymes that acetylate the 1- and 3-amino groups of the central deoxystreptamine ring and enzymes that acetylate the 2′ and 6′-amino groups of the primed, 6-deoxy-6-aminoglucose ring. These enzymes catalyze the acetylation of virtually all medically useful aminoglycosides i.e. gentamicin, tobramycin, netlimicin and amikacin. The most common aminoglycoside acetyltransferases in *P. aeruginosa* are AAC(3′) (3-*N* -aminoglycoside acetyltransferase) and AAC(6′) (6-*N*-aminoglycoside acetyltransferase) [171, 172]. AAC(6′)-II is the most common aminoglycoside acetyl transferase in *P. aeruginosa* [169] and confers resistant to tobramycin and gentamicin where as AAC(6′)-Ia is responsible for amikacin resistant in *P.* aeruginosa [173, 174]. Another AAC(6′)-II has been reported in few CF patients conferring tobramycin resistance [121].

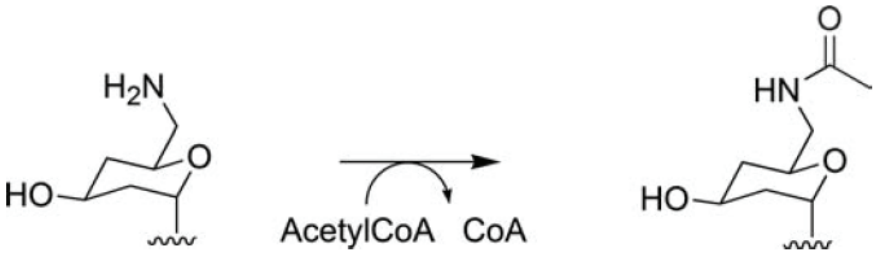

Figure 10. *Reaction catalyzed by acetyltransferases*

Aminoglycoside phosphotransferases (APH) are ATP-dependent kinases (30 kDa) which generate a phosphorylated aminoglycoside and ADP as products (***Figure 11***). The most prevalent group of aminoglycoside kinases are the APH(3′), which confer resistance to kanamycin and neomycin by phosphorylation of the 3′-OH and are commonly found in *P. aeruginosa* [170]. A chromosomal APH(3′)-IIb is reported by [125] responsible for insensitivity of *P. aeruginosa* to kanamycin [175]. Other APH(3′)s important in this organism are APH(3′)-VI conferring resistance to amikacin and isepamicin and APH(2′′) for gentamicin and tobramycin.

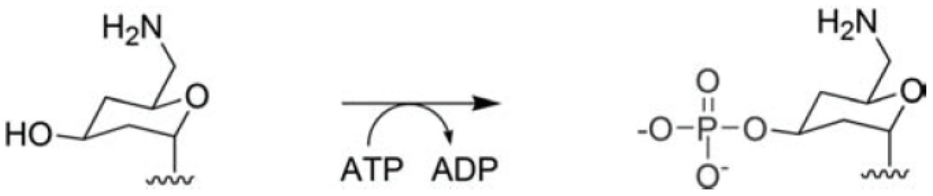

Figure 11*: Aminoglycoside modifying action of phosphotransferases*

Aminoglycoside nucleotidyltransferases (ANT) are the smallest group of aminoglycoside-modifying enzymes in terms of numbers; however they exhibit a significant impact on clinical aminoglycoside resistance. They catalyze the adenylation of aminoglycosides (***Figure 12***) such as streptomycin and gentamicin [176-178] in *P. aeruginosa*. ANT(2´´)-I is a major source of gentamicin and tobramycin resistance and with AAC(6´) and AAC(3) represents the most common enzyme dependant aminoglycoside resistance in *P. aeruginosa*. However ANT(2´´)-I does not have any effect on amikacin and netlimicin [177, 179]. Other subgroups nucleotidyltransferase are involved in aminoglycoside resistance in *P. aeruginosa* include streptomycin resistance conferring ANT(3´´) [168] and ANT(4´)-II for amikacin tobramycin and isepamicin resistance [163]. Two variants of ANT(4´)-II, ANT(4´)-IIa and ANT(4´)-IIb are encoded by chromosomal gene and/or plasmid in amikacin resistant clinical isolates [163].

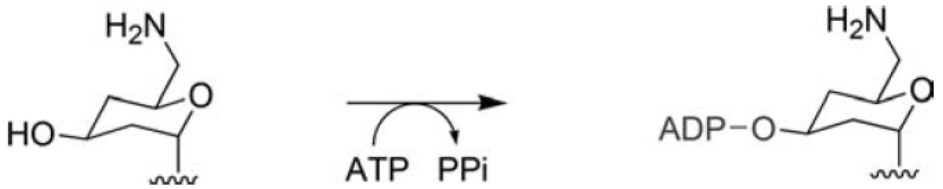

Figure 12*: Modification reaction catalyzed by nucleotidyltransferase*

1.10.4 Target modification

Aminoglycoside resistance through target modification can occur through two mechanisms; point mutation of rRNA or ribosomal proteins or methylation of the 16S rRNA. Point mutations in the 16S rRNA can result in resistance to aminoglycosides [180]. For example, for the 2-deoxystreptamine antibiotics (amikacin, gentamicin, neomycin), mutations of A1408 (*E. coli* numbering) confer high-level resistance [181, 182].

Antibiotics that incorporate the streptamine aminocyclitol such as streptomycin also bind to the codon-decoding site but make multiple contacts to 16S rRNA and proteins such as S12. Consequently, mutations in 16S rRNA and the ribosomal protein RpsL (S12) conferring high-level resistance that is clinically relevant in *Mycobacterium tuberculosis* [183, 184]. Similar mechanism has occasionally been demonstrated in *N. gonorrhoeae* and two other *Mycobacterium* species [181, 185]. Resistance based on target mutation is perhaps more rare in other cases since there are several copies of the 16S rRNAs and there is a minimum number of copies/mutations that must occur in order for resistance to be observed [181, 186].

High-level of resistance in aminoglycoside producers is frequently the result of ribosomal methylation usually catalyzed by S-adenosylmethionine-dependent methyltransferases that modify G1405 or A1408 of the 16S rRNA to the 7-methyl derivative [187]. The rRNA methylation typically confers very-high-level resistance to aminoglycosides compared to low or moderate level as seen in impermeability type resistance and until recently was confined to non-pathogenic actinomycetes. In 2003, a clinical isolate of *P. aeruginosa* was reported carrying 16S rRNA methyltransferase, RmtA, conferring resistance to all 4,6-disubstituted 2-deoxystreptamine such as gentamicin and kanamycin etc. [188]. This report was followed by identification of similar genes termed as *arm* from *S. marcescens*, *K. pneumoniae*, and *E. coli* [189-191]. The genes in *Enterobacteriaceae* are encoded on transposons and in *P. aeruginosa* on R-plasmids [192-194] facilitating dissemination. This mechanism aminoglycoside resistance determinant will therefore likely continue to spread among pathogenic *Pseudomonas* and possibly other Gram-negative bacteria.

1.11 GENETIC BASIS FOR RESISTANCE VIA CHROMOSOMAL CHANGES

The numerous genetic changes favouring the cellular physiology of resistance are complex and varied. Chromosomal mutations in common resistance genes, can be spontaneous or can be complex. The development of resistance due to chromosomal mutation for each drug is independent of the existing drug resistances. Resistance mechanism supported by chromosomal mutations holds implications to select treatment for individual patients. Bacteria that typically acquire chromosomal mutations to have stable resistance patterns in the short term; however, selective pressures in an individual patient will be an important factor to develop resistance over the long term. This relative stability allows the clinicians to test for resistance in a specific microorganism and modify antimicrobial therapy accordingly. Since the probability of developing multiple resistances mechanism in a specific species of bacteria in one patient is the product of the probabilities of developing each resistance individually. Therefore, a high load of the organism in the infected person is needed for multiple resistances to develop and treatment with multiple drugs may prevent the emergence of resistance.

2 AIMS OF THE THESIS

The general aim of this thesis was to provide a better understanding of the chromosomal mechanisms underlying resistance of three very important of antibiotics fluoroquinolone, carbapenems and aminoglycosides in two model Gram-negative organism *N. gonorrhoeae* and *P. aeruginosa*.

Specific aims

1. To determine the relative contribution of alterations in DNA gyrase, topoisomerase IV and gonococcal porin protein PorB1b for fluoroquinolone resistance in *Neisseria gonorrhoeae* (**Paper I**).

2. To investigate efflux mediated aminoglycoside resistance mechanism in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients **(Paper II)**.

3. To analyze the possible role of mutations in penicillin binding proteins, efflux pumps and the porin OprD in resistance to carbapenems in *P. aeruginosa* **(Paper III)**.

4. To elucidate the role of the efflux pump MexXY and other presumptive mechanisms of aminoglycoside resistance in long term antibiotic treated cystic fibrosis *P. aeruginosa* isolates (**Paper IV)**.

3 MATERIALS AND METHODS

3.1 BACTERIAL STRAINS:

Paper I:
Neisseria gonorrhoeae isolates were collected from ICCDR, B Dhaka, Bangladesh and described in paper [63] and were resistant to ciprofloxacin. In this study isolate 11 and 19 were used as donor strain A and B respectively. *N. gonorrhoeae* isolates were grown in chocolate agar (Oxoid Ltd., Basingstoke, UK) or in Tryptic Soya Broth (TSB) (Acumedia Manufacturers Inc., Baltimore, MD) at 37.0^0C in 5% CO_2. The strains were identified as oxidase positive Gram-negative diploccoci with sugar oxidation typical of *N. gonorrhoeae*. A ciprofloxacin sensitive clinical strain was used in this study as a recipient strain. The strains generated in this paper by transformation protocol are discussed in transformation section.

Paper II:
In this study, 15 aminoglycoside-resistant and 5 aminoglycoside-sensitive isolates were collected from different CF patients attending the CF centre Huddinge during 2001. The reference strain PAO1 (kindly provided by B.W. Holloway, Monash University, Melbourne, Australia) is used as a control strain. Laboratory derived mutants were produced by inoculating 10^8 PAO1 CFU on Iso-Sensitest agar (Oxoid Ltd., Basingstoke, UK) plates containing amikacin (Sigma-Aldrich, St. Louis, USA) in 4, 8, 16, 32 mg/L and incubated for 48 hours. To use as a positive control for the detection of aminoglycoside modifying enzyme AAC(6´)-Ib and ANT(4´)-IIb *P. aeruginosa* strains 101/1477, PPV-97 b, VR143/97 and *Acinetobacter baumanni* strain AC 54/97 (G.M. Rossolini, University of Siena, Italy) were used.

Paper III:
From a previous study done by El. Amin *et al.* 13 clinical *P. aeruginosa* strains were selected for carbapenem resistance during 2001-2003 at Karolinska Hospital Solna Stockholm Sweden [80]. Three of these isolates which were non beta-lactamase producing and resistant to imipenem were selected for conjugation experiments and further analysis. *P. aeruginosa* strain PAO18SR and PAO236 were used as recipient strain [195, 196].

Paper IV:
A total of 40 CF *P. aeruginosa* isolates were included in this paper including the isolates that were included in paper II. These *P. aeruginosa* isolates were all genetically different. The rest of the 20 isolates collected from six CF patients aged 27 to 33 at the CF centre Copenhagen, Denmark in two different time points 1994 (9 isolates) and 1997 (11 isolates). The Danish CF *P. aeruginosa* isolates were typed by PFGE (isolates form patients CF21, CF59, CF86 and CF89) or by ribotyping (isolates form patients CF166 and CF222) [197].

3.2 ANTIBIOTIC SUSCEPTIBILITY TESTING

In all paper MICs were determined by Etest (AB Biodisk, Solna, Sweden) which is a plastic strips containing predefined gradients of antibiotic concentration. The MICs were read in the intersection point of inhibitory eclipse according to the manufacturer's recommendation. In paper I, III and IV Muller-Hinton agar (BD Microbiology Systems, Cockeysville, MD, USA) was used and chocolate agar (Oxoid Ltd., Basingstoke, UK) was used in paper II as recommended by Clinical and Laboratory Standards Institute (CLSI).

3.3 SEROVAR DETERMINATION

Ph serovers and GS serovers of the *N. gonorrhoeae* isolates were determined by a coagglutination technique, using Phadebact Monoclonal GC kit (Boule Diagnostics AB, Huddinge Sweden) and genetic systems (Genetic systems Corp., Redmond, WA, USA) respectively.

3.4 PREPARATION OF CHROMOSOMAL AND PLASMID DNA

The chromosomal DNA from *P. aeruginosa* and *N. gonorrhoeae* isolates was extracted in all studies by QIAamp DNA mini kit (Qiagen, Hilden, Germany). The Plasmid DNA was prepared using QIAquick spin Minipreps according to the manufacturer's description. The DNA and plasmid preparation was checked on agarose gel electrophoresis and quantified using Nanodrop ND-100, Wilminngton, DE, USA). Chromosomal DNA or plasmid preparations were stored at -20^0C until used for further analysis.

3.5 EXTRACTION OF TOTAL RNA AND SYNTHESIS OF cDNA

Bacterial cells were grown at 37^0C in Luria Bertani broth for 16 to 18 hours and then the cells were diluted in a fresh culture (1:100) and were grown to the logarithmic phase (OD_{595} 0.5) and harvested by centrifugation at 2000g for 10 min at 4^0C. The supernatant was discarded and the cell pellet was resuspended in Tris buffer (pH 8.0). Approximately 10^8 cells were disrupted with lysozyme (Sigma) (1 mg/ml) and then subjected to RNA extraction. Total RNA extraction and purification were performed by using High Pure RNA Isolation Kit (Roche, Manheim, Germany) and stored at -70^0C until further used. RNA concentration was measured with the help of a spectrophotometer (NanoDrop ND 100). A total of 1.0 µg (in paper II and IV) or 0.5 µg (in Paper III) RNA from each isolates was used for reverse transcription reaction to produce cDNA using 1st Standard cDNA Synthesis Kit for RT-PCR (Roche). The cDNA was stored at -20^0C until used.

3.6 CONJUGATION OF *P. aeruginosa*

The horizontal transfer of genetic material between bacterial cells through direct cell-to-cell contact is defined as conjugation. The direct contact between the donor and recipient bacteria leads to establishment of a cytoplasmic bridge between them and transfer of part or all of the donor (contains specific conjugative plasmids) genome to the recipient. In our study only less than 10% of *Pseudomonas* chromosome was transferred and recombined between clinical *Pseudomonas* strains and strain PAO because of the presence of strong restriction system/s in PAO. To clarify genes contributing to carbapenem resistance other than the known genes or mechanisms we have used conjugation between carbapenem resistant clinical *P. aeruginosa* strains and a genetically well characterized strain (PAO). Because of the abundance of high frequency spontaneous mutations to imipenem resistance we selected auxotrophic nutrients marker in our study.

The conjugative plasmid R68.45 was transferred from *P. aeruginosa* PAO25 (R68.45) to the clinical strains by selection for kanamycin resistance. This plasmid was then transferred from clinical strains of *P. aeruginosa* to recipient strains i.e. PAO18SR (*proB64, pur-66*, strR rifR) and PAO236 (*ilv-226*, *his-4*, *lys-12*, *met-28*, *trp-6*, *proA*, *nalA*) [195]. The whole conjugation experiment was performed as described in [196] except that the recipient strain was grown at 42^0C to overcome restriction [196] with the clinical strains containing plasmid R68.45 as donors. Colonies were selected on minimal agar plates containing appropriate growth factors for the two markers of PAO18SR and the *proA* marker of PAO236. To prevent the growth of the donor strains streptomycin (1 g/L) and rifampicin (80 mg/L) was added for PAO18SR and nalidixic acid (1 g/L) for PAO236.Transconjugants that required either proline or adenine for growth and resistant to imipenem (2 mg/L) were selected for further studies. We have used a serotyping kit (Bio-Rad, Marnes-La-Coquette, France) to verify the transconjugants. Amino acids, adenine and all the antibiotics except imipenem were from Sigma-Aldrich, St. Louis, USA. Imipenem (Tienam®) was from Merck Sharp & Dohme (Sweden) AB, Sollentuna, Sweden.

3.7 TRANSFORMATION

The protocol that we have used to transform *N. gonorrhoeae* was adopted and developed from the protocol described by Goodman and Scocca and Anticgnac *et al.* [198, 199]. Whole cell DNA from donor *N. gonorrhoeae* strains was extracted using Qiagen DNA Mini Kit (Qiagen Inc.) according to the manufacturer's description. Recipient strains were grown on a chocolate agar plate at 37^0C for 18 hours in the presence of 5% CO_2 and rice-grain sized colonies of recipient strains were subcultured in 10ml Tryptic soy broth for another 18 hours in cell culture bottle maintaining same growth conditions. The whole-cell DNA from the donor strains were then added to the broth containing recipient *N. gonorrhoeae*. After 6 hours of incubation the bacterial culture was spun down, the supernatant was discarded and the cell pellets were resuspended in the remaining broth. A 150 µl of cell suspension was cultured in each

chocolate agar plate containing selected concentrations of ciprofloxacin. This protocol was used for controls in all transformation experiments without adding any donor DNA.

3.8 PCR AND DNA SEQUENCE ANALYSIS

The genes of interests were amplified by polymerase chain reaction (PCR) prior to sequencing. The PCR amplification was performed according to the standard protocol described for AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA) for all genes containing 1X PCR buffer, 200 µM of each dNTP, 1 pmol/µl of each primer, 1.5 mM $MgCl_2$, 1.5 U/50 µl of AmpliTaq DNA polymerase with proof reading activity and 5 ng of DNA form *P. aeruginosa* or 5 µl DNA from *N. gonorrhoeae* (Paper I). The master mix was aliquoted to a volume of 50 µl. The composition of the PCR mastermix was followed for all the genes included in this thesis except that 2% DMSO (dimethyl sulfoxide) was added in the master mix to amplify *mexZ* gene. All the reagents for PCR reactions were from Applied Biosystems except dNTP mix and DMSO were form Sigma (Sigma-Aldrich, St. Louis, USA).

The oligonucleotide primers (***Table 8***) used in this study were designed by either OLIGO 4.0 (National Biosciences Inc., Plymouth, MN, USA) or Primer Premier 5.0 (Premier Biosoft international, Palo Alto, CA, USA). The nucleotide sequence information for primer design was obtained either from Pseudomonas genome project (http://www.pseudomonas.com/) or from Genebank (http://www.ncbi.nlm.nih.gov/sites/entrez?db=genome). All the primers were synthesized by Thermo Hybaid, Ulm, Germany.

The DNA amplification was performed in a DNA thermal cycler, GenAmp PCR system 9700 (Applied Biosystems). The detail temperature profile for PCR reactions are described in Papers I-IV. Analysis of the PCR products or the purified templates prior to sequencing was performed on 1-1.5 % agarose (Invitrogen Corporation, Carlsbad, CA, USA) gels in TBE (Tris Borate EDTA) buffer at 100 volts for 30 minutes and stained with ethidium bromide followed by visualization under UV-Light. Molecular markers to compare the size of the PCR products were purchased from Promega (Promega Biotech AB, Stockholm, Sweden).

DNA sequencing of the PCR products was performed by dideoxy chain termination method [200]. At first the PCR products were purified from primers, free nucleotides and enzyme with the help of QIAquick-spin PCR purification Kit (QiaGen). Cycle sequencing reaction was performed using BigDye Terminator Ready Reaction Kit (Applied Biosystems) on the GeneAmp PCR System 9700 (Applied Biosystems). The extension products were purified by ethanol/sodium acetate/EDTA purification method as described by Applied Biosystems and then loaded into the ABI 310 genetic Analyzer

Table 8. *Oligonucleotide primers used in PCR/ Sequencing, pyrosequencing and realtime PCR*

Primer	Sequence	Annealing Temperatures	References
PCR & Sequencing			
qnr1	5′- GCTTTGGCATAGAGTTCAGG - 3′	43^0C-55^0C	Paper I
qnr2	5′- AGATCGGCAAAGGTTAGGTC - 3′		
LysR1	5′- CGATACGCCAATACGACCCG - 3′	51^0C	Paper I
LysR2	5′- GCATTGTTACCGAACCCTTGT – 3′		
LysR3	5′- GAAACGGCATCCAGTTCCTC - 3′	51^0C	Paper I
LysR4	5′- AGCCAGCCCACTTTGTCTATT - 3′		
mexZ-1	5′- AAGGGCGTGGGCACCACTGC - 3′	61.0^0C	Paper II
mexZ-2	5′- GGACCAGCGCAGGCACCTGA - 3′		
mexZ2p-U	5′- GGCGTTTCTGTAACATATCCTT – 3′	59.1^0C	Paper IV
mexZ2p-L	5′- GCGAGGAAGACGCCCAGC - 3′		
mexZS1	5′- GCGGGTGCTGGAGATCCT - 3′		Paper IV
mexZS2	5′- AGGATCTCCAGCACCCGC - 3′		Paper IV
mexOZ-1	5′- CAGCGAGCCGGTCCATTGGA - 3′	60.0^0C	Paper II & IV
mexOZ-2	5′- TCGCACATCGCCAGGCAGAC - 3′		
AAC6IbU	5′-GAG TGG GGC GGA GAA GA- 3′	58.0^0C	Paper II & IV
AAC6IbL	5′- CCC AAG CCTTTGCCCAGTTG- 3′		
ANT4IIBU	5′- TTACCGCACCTGGATAGAGC- 3′	58.0^0C	Paper II & IV
ANT4IIBL	5′- GATGGGGATCAACAATGTCG - 3′		
APHIIB-1	5′- ATGCATGATGCAGCCACCTCCAT - 3′	56.0^0C	[201]
APHIIB-4	5′ - CCTACTCTAGAAGAACTCGTCCA - 3′		
galUF140-U	5′- CGAGCGCAGCCTGATTAGACT - 3′	61.0^0C	Paper IV
galUR645-L	5′- GGAGCAGCG GAACTGGTTGTA - 3′		
galUF565-U	5′- AGCCGTTCGCCGTGGT - 3′	61.0^0C	Paper IV
galUR1121-L	5′- ACAGCTCAGGTAGGGCGGATA - 3′		
rplYF144-U	5′- ATCGCCCGAACGCTGGT - 3′	61.0^0C	Paper IV
rplYL902-L	5′- ATGCCGGGTCTGGTCGTATTC - 3′		
pbp-1b-f	5′ - TCGTGACCAATCCGGAAAC - 3′	61°C	Paper III
pbp-1b-r	5′- GCGGTGGACAGGTTGTAGGAG - 3′		
pbp-3-f	5′ - TACCTGGCTCATCGCGAACTG - 3′	61°C	Paper III
pbp-3-r	5′ - GGATGCCGGTGAGATCGAG - 3′		
pbp3-seq1	5′ - CCTGAAGGTGCCCGGCGTGTA - 3′		Paper III
pbp3-seq2	5′ - ACCCTGCAGATCGGCCGCTAC - 3′		Paper III
pbp-2-a-f	5′ - ATGCCGCAGC CGATCCACCT - 3′	62°C	Paper III
pbp-2-a-r	5′ - TTACTGTTCAAGGGCGGGCG - 3′		
pbp2-seq-1	5′ - CTGGCGATGGTCAGCCAGCC - 3′		Paper III
pbp-2-seq-2	5′ - GAAGCTGATGCCGGTGACGA – 3′		Paper III
pbp-6-a-f	5′ - ACAGCATCCGCGTGGC - 3′	63°C	Paper III
pbp-6-a-r	5′- ATCGCGTAGTGGCTCGGCTCA - 3′		
oprD-a-f	5′ – ATGAAAGTGATGAAGTGGAGC - 3′	58°C	[80]
oprD-a-r	5′ - AGGGAGGCGCTGAGGTT- 3′		

Table 8. *Continued*

Primer	Sequence	Annealing Temperature	Reference
PCR & Sequencing			
oprD-b-f	5′- AACCTCAGCGCCTCCCT – 3'	58°C	[80]
oprD-b-r	5′ - ATACTGACCTCTCCTGTTCG - 3'		
Pyrosequencing			
TRDR-U	5′-TCAGAATGTCACGGTGAATAC - 3′	56.0^{0}C	Paper IV
TRDR-L	5′- BIOTIN -TTCGTGTGGGAGCTTATGA -3′		
TRDRSeq-1	5′- CTTGTACACACCGCCCGT - 3′		Paper IV
TRDRSeq-2	5′- GTTACCACGGAGTGATTC - 3′		Paper IV
Realtime PCR			
rpsL-1	5′- GCTGCAAAACTGCCCGCAACG - 3′	60^{0}C or 62^{0}C	Paper II, III, IV
rpsL-2	5′- ACCCGAGGTGTCCAGCGAACC - 3′		
mexY-1	5′- GGACCACGCCGAAACCGAACG - 3′	62^{0}C	Paper II
mexY-2	5′- CGCCGCAACTGACCCGCTACA - 3′		
mexB-1	5′- CAAGGGCGTCGGTGACTTCCAG - 3′	62^{0}C	[80]
mexB-2	5′- ACCTGGCAACCGTCGGGATTGA - 3′		
mexZ-1	5′- AGGTCTGCCTGGCGATGTGC- 3′	62^{0}C	Paper II
mexZ-2	5′- AGCGTTGCCCCTGCTTCTCG – 3′		
oprD-1	5′- CGACCTGCTGCTCCGCAACTA - 3′	62^{0}C	Paper II
oprD-2	5′- TTGCATCTCGCCCCACTTCAG- 3′		
oprD1-f	5′ - CGA CCT GCTGCTCCGCAACTA - 3′	60^{0}C	[80]
oprD1-r	5′ - TTGCATCTCGCCCCACTTCAG - 3′		
oprM-1	5′- CGGATCGGCGTGGACGGTAG -3′	62^{0}C	Paper II
oprM-2	5′- GGTGCCCAGGGTGTCCTTGG - 3′		
nuoHF657-U	5′- GCAGGAACTGGCGGACGG - 3′	62^{0}C	Paper IV
nuoHL823-L	5′- GGTCTTGGCGGCGAAGTAGAA - 3′		
nuoNF148-U	5′- CTGTCGCTGCTGCCGGTCCTC – 3′	62^{0}C	Paper IV
nuoNL297-L	5′- TACAGCTCTTCGCGGTTACCC – 3′		
PA5471-U	5′- CGACATCGGCTGTGGCA - 3′	62^{0}C	Paper IV
PA5471-L	5′- AGTCGCTCCAGGTCTCGTC - 3′		
pbp-2 –f	5′ - GCCCAACTACGACCACAAG - 3′	62^{0}C	Paper III
pbp-2 –r	5′ - CGCGAGGTCGTAGAA ATA G - 3′		
pbp3-f	5′ - TGATCAAGTCGAGCAACGTC - 3′	62^{0}C	Paper III
pbp3-r	5′ - TGCATGACCGAGTAGATGGA - 3′		

(Applied Biosystems). Both strands of PCR amplified fragments were sequenced twice but no errors in PCR amplification or sequencing were detected. Nucleotide and deduced amino acid sequences were analyzed by using Finch TV (www.geospiza.com), ClustalW Interactive Multiple Sequence Alignment at European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/clustalw2/), UK and ExPaSy Molecular Biology

Server at Swiss Institute of Bioinformatics (http://www.expasy.ch/tools/dna.html), Geneva, Switzerland.

3.9 QUANTITATIVE OR QUALITITATIVE ANALYSIS OF RESISTANCE GENES BY REALTIME PCR

Realtime PCR is a technique that allows simultaneous amplification and detection of targeted DNA in real time by the help of fluorescent molecule. To analyze gene expression quantitatively or qualitatively at first the extracted mRNA has to be converted into cDNA. In all the papers SYBR green based realtime PCR assay had been used. SYBR green is a fluorescent dye which binds to the double stranded DNA. In its unbound form it produces relatively low fluorescence but its fluorescent significantly increased when it is bound to the double stranded DNA. The increase in fluorescence is measured in every cycle of an ongoing PCR reaction and registered. When in a certain cycle number the amount of fluorescence increases over the back ground i.e. threshold level reflecting the amount of starting amount of nucleic acid template in a reaction which is defined as the crossing point (Cp) value. The amount of starting nucleic acid material and the Cp value are inversely related thus allow realtime PCR method to quantify gene expression. As SYBR green binds to both specific and non specific DNA the specificity of a realtime PCR reaction is measured by performing a melting curve analysis just after the completion of the PCR reaction. The specific PCR product will have a specific melting pick distinguishing it from other non specific amplification if any present. This allows identifying specific amplification of certain gene which in turn permits to use realtime PCR method for qualitative purposes.

mRNA expression of *mexB*, *mexY*, *mexZ*, *oprM*, *oprD* and PA5471 was measured by quantitative realtime PCR on a Lightcycler™ (Roche) followed by standard protocol described in LightCycler-FastStart DNA Master SYBR Green I Kit (Roche). Realtime PCR assay was also used to asses the normal expression of *nuo* operon in a qualitative manner by analysing the melting curve in the LightCycler DNA analysis Software (Roche). For both quantitative and qualitative analysis realtime PCR was done as duplicate (Paper II & IV) or triplicate (Paper III) using different cDNA preparation to analyse any specific gene expression. All the primer sequences **(*Table 8*)** used in realtime PCR assay are from Pseudomonas (http://www.pseudomonas.com) database. The ribosomal protein S12 gene *rpsL* was used as internal control. The mRNA levels of a specific gene were expressed by comparing with the expression of the internal control on that strain and also in PAO1 and then the expression values were calculated based on a standard curve, where 3.4 cycles caused a 10 fold increase in cDNA. A strain was considered to hyperproduce mRNA for pump proteins if cDNA level was >5 times that of PAO1. In Paper III the realtime PCR data was assessed by using a Microsoft ™ Excel sheet using the Pfaffl equation [202] followed by statistical analysis with STATISTICA software (StatSoft Inc., Tulsa, OK).

3.10 PYROSEQUENCING

Pyrosequencing™ is a one-step, gel-free, sequencing-by-synthesis method where nucleotide incorporation proceeds sequentially along each DNA template at a given nucleotide dispensation order (NDO). Each nucleotide is dispensed and tested individually for its incorporation into a nascent DNA template. Pyrosequencing is catalyzed by four kinetically well-balanced enzymes, DNA polymerase, ATP sulfurylase, luciferase, and apyrase. Each incorporation event is accompanied by release of pyrophosphate (PPi) in a quantity equimolar to the amount of nucleotide incorporated. ATP sulfurylase quantitatively converts PPi to ATP in the presence of adenosine 5' phosphosulfate. ATP then drives the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light in amounts that are proportional to the amount of ATP. The light is detected by a charge coupled device (CCD) camera and displayed as a peak in a pyrogram™. Each peak height is proportional to the number of nucleotides incorporated. Unincorporated dNTP and excess ATP are continuously degraded by Apyrase. After the degradation is completed, the next dNTP is added and a new Pyrosequencing cycle is started. As the process continues, the complementary DNA strand is built up. Nucleotide sequence (70-100 nt) is determined from the order of nucleotide dispensation and peak height in the pyrogram.

Pyrosequncing was carried out according to the standard protocol advised by the manufacturer with slight modification. In brief, 2 ng/ µl of chromosomal DNA was used as a template to amplify a 220 nucleotide fragment from the 3´ end of 16S rRNA using PCR primer TRDR-U and TRDR-L-biotin **(*Table 8*)** as described above. The amplified fragment held the sequence for the A site of 16s rRNA. Twenty six microliter of this PCR product was mixed with 20% streptavidin-Sepharose dissolved in 26 µl of binding buffer and incubated at 25^0C for 10 minutes. To capture the streptavidin-biotinylated template complex the whole mixture was transferred to a cellulose nitrate (NUNC A/S, Roskilde, Denmark) filter plate and was incubated with 0.5 M NaOH (50ul each well) for 1 minute. The non-biotinylated DNA strand and NaOH solution were washed away by the help of a vacuum pump and then washed twice with 150 µl 1 x annealing buffer (200mM Tris-acetate and 50mM Mg-acetate). Primer TRDR-1 was used as a sequencing primer for the nucleotide position 1399 to 1415 and TRDR-2 for nucleotide position 1484 to 1492 of the A site of the 16S rRNA genes. These primers were hybridised separately to the single stranded PCR product by adding 0.35 µM of sequencing primer in 45 µl annealing buffer to each well. After gentle mixing the whole mixture was transferred to a pyrosequencing plate and then incubated for 2 minutes at 90^0C. The pyrosequencing reaction was performed using 96 SQA reagent kit containing enzyme and substrate mixture and the 4 nucleotides as provided by Pyrosequencing (Pyrosequencing AB, Uppsala, Sweden). The result of pyrosequencing was accepted based on the quality ratings by the provided software and then analysed visually. A presence of double or multiple peaks for single nucleotide position is considered as a point mutation in any of the 4 copies of 16S rRNA gene.

4 RESULTS AND DISCUSSION

4.1 CIPROFLOXACIN RESISTANCE IN *N. gonorrhoeae* (PAPER I)

The impressive ability of *N. gonorrhoeae* to develop resistance against various antibiotics is probably the most remarkable reason for the rapid increase of ciprofloxacin resistance worldwide during the last few years [203, 204]. There are numerous reports of the correlation between *gyrA* mutations with or without additional alterations in QRDR of *parC* and a Pro439 to Ser mutation in *parE* has been reported. [53, 63, 74, 205]. *N. gonorrhoeae* has known efflux systems as MtrCDE (Mtr: multiple transferable resistance), NorM, MacA-MacB and Far but none of them seems to export ciprofloxacin as a preferred substrate [53]. Alterations only in QRDR of *gyrA* seem to generate MICs of ciprofloxacin at levels of 0.125 - 0.5 mg/L [65]. Clinical strains, resistant to ciprofloxacin, exhibit a wide range of MIC from 0.125 to >32, implicating additional mechanisms of resistance, other than alterations in the target enzymes Transformation study has been taken under consideration to determine if alterations in gonococcal porin PorB1b could be involved in resistance to ciprofloxacin. Four separate transformation experiments were performed and transformation rate in the experiments were ca 1/1000 to 1/10000. Alterations in LysR-family transcriptional regulator (putative *lysR*) and presence of *qnr* gene have also been investigated.

4.1.1 Transformation, transfer of chromosomal resistance determinants

Transformation experiments #1 and #2: Two transformants from transformation #1, MIC of ciprofloxacin 0.064 mg/L, had only one of the alterations in GyrA position 95 and the other ten transformants from transformation #1 and all eight transformants from transformation #2 had alterations in GyrA positions 91 and 95 compatible with incorporation of DNA from the donor strains, with MICs of ciprofloxacin of 0.125 - 0.25 mg/L. Ph serovars and GS serovars of all tested transformants were the same as the ones of the recipient(***Table 9***).

Transformation experiments #3 and #4: Donors were extracted DNA from strains A and B respectively, and recipient was transformant 1A11, here renamed to strain 2. MIC of ciprofloxacin of the recipient was 0.25 mg/L. All transformants that were selected for further investigation were shown in ***Table 10***. Three transformants from transformation #3 with MIC of ciprofloxacin 2-4 mg/L had no alteration in ParE and one transformant with MIC of ciprofloxacin 8 mg/L had a Pro to Ser alteration in position 439, same alteration as the donor strain ParE [63]**.** Most transformants had changed the serovars.

4.1.2 DNA sequence analysis

The sequences of *porB1b* in the analyzed transformants were similar to their recipient strains in transformation 1 & 2. But the sequences *porB1b* in transformation 3 and 4 were identical to the donor strains (***Table 9 and 10***). The gene *qnr* was not detected by PCR in strains A, B, or any of the two other ciprofloxacin highly resistant

N. gonorrhoeae strains and the putative *lysR* sequence in the transformants 1A11, 1B15, 2A26 and 2B27 were similar to donors A and B and recipient strain 1.

Table 9. *Transformation experiments #1 and #2: N. gonorrhoeae ciprofloxacin susceptible strain 1 was used as recipient and extracted DNA preparations from ciprofloxacin highly resistant N. gonorrhoeae strain A and B were used as donors*

Strain	MIC CIP (mg/L)	*gyrA* Ser91	*gyrA* Asp95	*porB1b* Position1 20	*porB1b* Position 121	Serovars	
A (donor)	>32	Phe	Gly	Lys	Asp	IB-1	Bropt
B (donor)	32	Phe	Gly	Lys	Gly	IB-3	Bopyst
1 (recipient)	0.008	Ser	Asp	Gly	Gly	IB-24	Bx
Transformation 1							
1A15, 1A16	0.064	Ser	Gly				Bx(1^1)
1A23, 1A14, 1A29, 1A210	0.125	Phe	Gly				Bx (2^1)
1A11, 1A19, 1A21, 1A22, 1A27, 1A25	**0.25**	Phe	Gly	Gly (1^1)	Gly (1^1)	IB-24 (1^1)	Bx (2^1)
Transformation 2							
1B21, 1B14, 1B19, 1B16	0.125	Phe	Gly				Bx (1^1)
1B15, 1B13, 1B10, 1B17	0.25	Phe	Gly	Gly (1^1)	Gly (1^1)	IB-24 (1^1)	Bx (2^1)

CIP, ciprofloxacin; MIC, minimum inhibitory concentration, [1] Indicates number of strains tested in the group.

4.1.3 Involvement of PorBIb in FQ resistance in *N. gonorrhoeae*

It has been stated in several reports that changes in porin composition or production could be a reason of antibiotic resistance mechanism in microorganisms. One notable example is the loss of outer membrane porin protein OprD in *P. aeruginosa* which results in imipenem resistance [80]. Higher affinity for *E. coli* outer membrane protein OmpF was demonstrated for newer quinolones with better activity against Gram-positive bacteria. However this report did not include ciprofloxacin resistant strains [206]. In *K. pneumoniae* loss of outer membrane protein OmpK36 is associated with a moderate increase in fluoroquinolone resistance in strains with target alterations or active efflux [207].

It is worth to mention that there is not so much information about PorB related ciprofloxacin susceptibility in *N. gonorrhoeae* and the absence of a defined wild type *porB* in *N. gonorrhoeae* perplexes the conclusive correlation and ciprofloxacin resistance. Corkill *et al.* have shown a reduced uptake of ciprofloxacin in a resistant strain and transformation of resistance to other strains, but neither the resistant strain

nor the transformants were sequenced in *gyrA* and resistance to chloramphenicol and tetracycline was not co transformed [208]. Moreover the uptake and efflux was not conclusive and done from only 10 strains [71].

Table 10. *Results of transformation experiments #3 and #4: N. gonorrhoeae ciprofloxacin moderately resistant strain 2 (transformant 1A11 from exp. #1) was used as recipient and extracted DNA preparations from ciprofloxacin highly resistant N. gonorrhoeae strain A and B were used as donors.*

Strain	MIC CIP (mg/L)	*parE* Pro439	*porB1b* Position 120	*porB1b* Position 121	Serovars	
A (donor)	>32	Ser	Lys	Asp	IB-1	Bropt
B (donor)	32	Ser	Lys	Gly	IB-3	Bopyst
2=1A11 (recipient)	0.25	Pro	Gly	Gly	IB-24	Bx
2A29	0.5					Bsx
2A27, 2A17	1.0					Bsx
2A15, 2A19	2.0					Bx
2A22, 2A28	2.0	Pro				Bsx
2A13, 2A21	2.0	Pro				Bropst
2A23	2.0					Bopst
2A14, 2A16	4.0	Pro				Bsx
2A110, 2A31, 2A32, 2A12, 2A18	4.0					Bopst
2A24	4.0	Pro	**Lys**	**Asp**	IB-9	Bopyst
2A26	8.0	Pro				Bvx
2A210	8.0	**Ser**				Bsx
2A25	16					Bsx
2B11, 2B13, 2B15, 2B18, 2B19, 2B110	0.5					Bsx
2B17	0.5					Bx
2B12	1.0					Bx
2B28, 2B22	1.0					Bsx
2B21, 2B27	2.0		Lys (1[1])	Gly (1[1])	IB-3 (1[1])	Bpyst
2B23	2.0					Brpyst
2B25	2.0					Bopyst
2B29	2.0					Bsx
2B26	2.0					Bropstx

[1] Indicates number of strains tested in the group if less than all

The genomic locus *penB* is equivalent to alterations in PorB1b loop three, i.e. Gly101 to Asp and Ala 102 to Asp, reducing PorB1b permeability to hydrophilic antibiotics as penicillin and tetracycline in *N. gonorrhoeae*. [209]. Furthermore chromosomally-mediated penicillin and tetracycline resistance is contributed by the *penB* equivalent

mutations in *porB1b* loop three in position Gly120 and Ala 121, i.e. to single mutation Gly120 to lysine or double mutations to charged amino acids. However from Genebank studies they stated that Lys and Asp mutations in position 120 and/or 121 occur in nature in *N. gonorrhoeae* [210]. Despite the different position numbers, these amino acids are likely to be the same since Gill *et al.* probably removed the 19 amino acid long signal sequence from their numbering. Besides, Olesky *et al.* did not include ciprofloxacin susceptibility as an aspect in their study. Donor strains A and B, used in paper I, have the 120Lys 121Asp and the 120Lys 121Gly sequence respectively, and both were ciprofloxacin resistant. The gene *porB* is known to be highly variable in sequence that includes loop three [211, 212].

There are a few reports on PorB and ciprofloxacin susceptibility in *N. gonorrhoeae.* In one study it has been shown that changes from wild type PorB in ciprofloxacin resistant strains of *N. gonorrhoeae* have been found in loop 3, Gly120 to Asn and Ala121 to Asp, as well as changes in loop 5. This report originated from a study of an outbreak which implicated that the studied isolates were related [72]. However, in another study no correlation between amino acid substitutions in PorB position 120 and 121 and resistance to fluoroquinolones was found from 33 *N. gonorrhoeae* strains [74]. This implicates that the relation between PorB and ciprofloxacin resistance may be more complex than specific changes in position 120 and 121. In paper I, the whole *porB1b* genes were transformed and MIC of ciprofloxacin increased from 0.25 to 0.5 - 16 mg/L and 0.5 - 2 mg/L respectively in the second generation transformants. In transformation studies we have compared the sequence of donor and recipient strains which allow us to be less dependent on a defining wild type sequence.

The possibility of co-transformation of another gene along with *porB* was also assessed, which might be responsible for fluoroquinolone resistance. The genome was searched up and down-stream of *porB* and only a putative LysR family transcriptional regulator was found the next gene downstream to *porB*, as a possible regulator on an efflux system. However, no alterations were found in *lysR*, in donors, recipients or transformants which implicates that LysR is not involved as fluoroquinolone resistance in these strains. In addition, *porB* was transformed repeatedly in two different transformation experiments, which addresses the preference of this gene being the one that confers resistance.

This study supports the fact that two mutations at position 91 and 95 of GyrA QRDR correspond to MIC of ciprofloxacin of 0.064 to 0.5 mg/L [58, 63]. Furthermore a linear relationship exists between the presence of mutations in QRDR of *gyrA* and *parC* and ciprofloxacin resistance. From transformation experiments #3 and #4 it has been found that a second transformation with strain A generated transformants with MIC of ciprofloxacin 0.5 - 16 and 0.5 - 2 mg/L respectively, implicating that further mechanisms of resistance were transformed in transformation #3. One explanation might be mutations in *parE* [63]. In transformation experiment #3 we found an alteration in ParE in transformant 2A210 with MIC of ciprofloxacin 8 mg/L, but not in transformants with MIC of 2 and 4 mg/L. Mutation in *parC* contribute to

fluoroquinolone resistance in Gram-negative bacteria but it was not possible to asses their role in *N. gonorrhoeae* in paper I as both the donor strains had wild-type *parC* sequence.

Protection has been involved in ciprofloxacin resistance in other species [54, 213, 214], by the plasmid gene *qnr*. Since the gene *qnr* was not found in any of the donor strains we excluded this mechanism of resistance was introduced in our strains.

In paper I it has been shown that *N. gonorrhoeae* transformants comprising donor *porB1b* also had increased MICs to ciprofloxacin, which is in notion with the fact that an alteration in outer membrane protein reduces the uptake hydrophilic antimicrobial agents. The PorBIb mediated resistance to ciprofloxacin in *N. gonorrhoeae* seems mainly to be of importance in combination with alterations in the target enzymes preferably GyrA, and can explain some of the wide range of MIC of ciprofloxacin exhibited by resistant strains.

4.2 CARBAPENEM RESISTANCE MECHANISMS IN *P. aeruginosa* (Paper III)

4.2.1 Conjugation study and transferrable resistance mechanism to carbapenems

Transconjugants, auxotrophic for at least one of the markers of PA018SR or PA0236, with the same serotype as the PAO strains and growing on imipenem-containing plates were selected and their MICs for imipenem, meropenem, ciprofloxacin and ceftazidime were determined, (***Table 11***). Of the 13 selected clinical strains, only 3 produced imipenem-resistant transconjugants after selection for *proB* in PA018SR: CG1, CG2 and CG13. The imipenem MICs of the transconjugants ranged between intermediate (>4 mg/L) to highly resistant to (>32 mg/L). Some transconjugants were also intermediately resistant to meropenem. The purine marker of PA018 or the *proA* marker of PA0236 gave no imipenem-resistant recombinants.

4.2.2 Sequencing of chromosomal genes and involved in carbapenem resistance

The active sites of the PBPs located close to both markers were sequenced. The SXXK box holding the active site serine was the focus. The PBPs that were sequenced were *ponB* (corresponding to PBP-1b) and *pbpB* (PBP-3), located close to *proA*. *pbpA* (PBP-2) and *dacC* (PBP6) were also sequenced. However, no mutations were found in the clinical strains or in the transconjugants sequenced that could explain imipenem resistance. All the strains sequenced for the SXXK box remained unchanged compared with their wild-type counterpart for PBP1b (468-SLIK-471), PBP2 (326-STVK-329 and 203-KTG-205), PBP3 (293-STVK-296) and PBP6 (64-STVK-67). The clinical strains had some alterations in the nucleotide sequence outside the active site

SXXK that did not alter the respective amino acid sequence. The penicillin-binding proteins PBP2 and PBP3 have been reported to be involved in carbapenem resistance in *E. coli* [81]. In order not to miss mutations outside the conserved region that could have an impact on the whole amino acid sequence or the active site, the complete *pbpA* (corresponding to PBP2) genes of strains PA018, CG13 and transconjugants 1c and 13c were sequenced. The entire *pbpB* (PBP3) gene of strains PA018, CG13 and transconjugant 13c were also sequenced. In *pbpA* sequencing, the clinical strain CG13 had a deletion of amino acid valine from position 28, leaving the whole open reading frame unchanged. In both *pbpA* and *pbpB* sequencing, other alterations in clinical strain CG13 were found, resulting in no amino acid changes. In a study by Legaree *et al.* it was shown that mecillinam at concentrations between 200 mg/L and 400 mg/L as well as mutations in *pbpA* cause spherical cells [107]. In our study, absence of morphological changes in the recombinants growing in the imipenem restriction zone in the Etest (data not shown) also indicates lack of alterations in PBP2. Since alterations in the OprD porin can cause imipenem resistance [85, 215], we sequenced the *oprD* gene for clinical strains and transconjugants (***Table 12***). All of the clinical isolates had mutations that could explain some of their resistance patterns; however the transconjugants had the same sequence as PA018SR, demonstrating that the *oprD* gene was not transferred during conjugation.

Table 11. *Minimum inhibitory concentrations (MICs) of clinical strains and their transconjugants*

Strain/ Transconjugant	Serotype	MIC (mg/L) [a]			
		Imipenem	Meropenem	Ceftazidime	Ciprofloxacin
PA018	PME	0.75	0.38	1	0.094
CG1	PMA	32	4	1,5	0.125
1a	PME	24	2	0.5	0.64
1b	PME	24	1.5	0.5	0.64
1c	PME	32	2	0.5	0.64
CG2	PMA	16	2	1	0.125
2a	PME	>32	4		
2c	PME	24	4		
2e	PME	32	6		
2f	PME	32	4		
2g	PME	24	2		
2h	PME	4	1		
CG13	NT	32	1	2	0.016
13a	PME	16	2	1	0.064
13c	PME	24	2	1.5	0.64
13e	PME	24	1.5	1	0.064
13f	PME	24	2	1	0.064

NT; not typeable; [a] MIC breakpoints according to European Committee on Antimicrobial Susceptibility Testing (EUCAST): imipenem, susceptible (S) ≤4 mg/L, resistant (R) >8 mg/L; meropenem, S ≤2 mg/L, R >8 mg/L; ceftazidime, S ≤8 mg/L, R >8 mg/L; and ciprofloxacin, S ≤0.5 mg/L, R >1 mg/L

Table 12. *Sequencing of the oprD gene*

Strain/ transconjugant	MIC (mg/L)		Amino acid position and substitutions																								
	IMP	MER	59	73	115	170	184	185	210	240	262	276	281	296	301	310	315	340	347	372	373–383	432					
PA018	0.75	0,34	S	Y	K	F	E	P	I	S	N	A	A	K	Q	R	A	Q	L	M		F					
CG1	32	4			T	L																					
1c	32	2																									
CG2	16	2	R	Stp *																							
CG13	32	1					Q	G	A	T	T	A	G	Q	E	G	G		M	V	Del/Ins **						
13c	24	2																									

MIC; minimum inhibitory concentration, *Stop codon, ** Deletion and insertion, frame restored at amino acid position 384

4.2.3 Transcription level of chromosomal genes in carbapenem resistance

The mRNA expression of *oprD, pbpA, pbpB* and *mexB* was measured using realtime PCR (***Table 13***). The expression of *oprD* was downregulated in all of the clinical strains and in all transconjugants. Expression of PBP2 and PBP3 (***Table 13***) was decreased in all strains compared with PA018SR, except for the clinical strain CG1 with a clear increase in both PBPs. The resistance pattern of CG1 and its transconjugant 1c were very similar (***Table 11***), so the increased expression of the genes for PBP2 and PBP3 is probably not important for resistance to carbapenems. The expression of *mexB* mRNA was slightly downregulated in two transconjugants.

Table 13. *Realtime polymerase chain reaction results (mean value)*

Strain/transconjugant	MIC (μg/mL)		Gene transcription (fold PA018)			
	IMP	MER	OprD	PBP2	PBP3	MexB
PA018	0.75	0.38	1.00	1.00	1.00	1.00
CG1	32	4	0.03	9.25	6.08	0.91
1c	32	2	0.003	0.67	0.6	0.14
CG13	32	1	0.06	0.57	0.64	1.62
13c	24	2	0.25	0.76	0.68	0.23
13g	16	2	ND	0.39	0.2	ND

MIC, minimum inhibitory concentration; IMP, imipenem;
MER, meropenem; ND; not done

Overexpression of genes for the MexAB-OprM efflux pump contributes to multidrug resistance, including meropenem. Since the clinical strains and transconjugants had increased MICs of meropenem, we determined the expression of *mexB*, which was not significantly altered in all strains analysed (***Table 13***) and thus not involved in the meropenem resistance observed in the studied transconjugants. It has been reported that AmpC β-lactamase alone has a very slight effect on intrinsic resistance to penem antibiotics but when combined with MexAB-OprM efflux system it plays a considerable role in clearing of penems [94]. The clinical strains and transconjugants that were selected for paper III had slow reactivity (>10 s) to nitrocefin (data not shown), indicating a normal or low level of AmpC β-lactamase expression as well as other β-lactamases [80]. When combined with a low level of MexB, we concluded that AmpC β-lactamase and/or MexAB-OprM efflux system are not involved in meropenem resistance in the studied transconjugant strains.

The most important mechanism of resistance to imipenem in clinical strains is decreased production of OprD, and loss of OprD raises the imipenem MICs from 1–2 mg/L to 8–32 mg/L [78]. Decreased transcription of *oprD* was found both in clinical strains and in transconjugants, explaining imipenem resistance and probably also the increase in the MIC seen for meropenem. This indicates that downregulation of the porin gene alone is enough to induce high-level imipenem resistance. OprD is regulated by multiple systems and is repressed by salicylates, subject to catabolite repression, and activated by arginine/ArgR and a variety of other amino acids [92]. MexT (PA2492) is a transcriptional repressor that downregulates *oprD* and upregulates genes for the efflux pump MexEF-OprN (so-called *nfxC* class mutants). The MexEF-OprN efflux pump mediates resistance to several antibiotics, including quinolones. *mexS* (PA2491) and *mvtA* have similar effects [106, 110], but none of them are close to the *proB* marker. None of our recombinants was resistant to ciprofloxacin, indicating that these regulator genes were not affected; also, any of the clinical strains did not show significant increase of *mexF* mRNA expression [80].

The imipenem resistant clinical strains had wild-type sequence for PBP1b, PBP2, PBP3 or PBP6. However, selection for the *proB* marker in PA018 resulted in the downregulation of *oprD* in imipenem-resistant transconjugants. This finding indicates that one or more regulatory genes for *oprD* are located close to the *proB* gene (PA4565 at 5113 kb).

4.3 AMINOGLYCOSIDE RESISTANCE MECHANISMS IN *P. aeruginosa* (PAPER II & IV)

Aminoglycoside resistance in clinical isolates from CF patients occurs mainly due to reduced drug uptake or accumulation as a result of impermeability type resistance and/or by adaptive resistance to the antibiotic. *P. aeruginosa* strains colonizing cystic fibrosis lungs undergo treatments with different combinations of antibiotics over the years and they have a general tendency to undergo clonal expansion to select for higher MICs of antimicrobials. In case of aminoglycoside antibiotics, this increase in MICs is usually due to chromosomal changes rather than acquiring genetic elements

by means of horizontal transfer of genetic elements *i.e.* plasmids [120, 121].Paper II and IV in this thesis concentrate on the chromosomally mediated aminoglycoside resistance mechanisms. To focus more on the genetic changes we have studied *P. aeruginosa* amikacin resistant in laboratory mutants, CF isolates from different patients and also isolates from same CF patients collected from two different time points in three years interval. In addition, several reported chromosomal non-enzymatic aminoglycoside resistance mechanisms have also been discussed to elucidate the main resistance mechanism in CF isolates from Nordic countries.

4.3.1 Isolation of *P. aeruginosa* mutants (Paper II)

Eight single colonies were isolated from each plate and were checked for minimal inhibitory concentration (MIC). The mutants investigated were chosen from several independent experiments. The frequency of amikacin-resistant mutants was ca 10^{-7}.

4.3.2 Antibiotic susceptibility pattern of cystic fibrosis *P. aeruginosa* isolates (CFPA) and amikacin resistant laboratory mutants (Paper II & IV)

The antibiotic susceptibility of the *P. aeruginosa* mutants (Paper II) is shown in ***Table 14***. The MICs of amikacin for the mutants were 4 - 64 mg/L and for wild type 2 mg/L. All mutants showed elevated MIC of amikacin (2 to 32 fold) and netlimicin (2 to 16 fold). There was no remarkable increase in MIC of tobramycin and gentamicin. All mutants except AK67 were susceptible to ceftazidime, meropenem, imipenem, norfloxacin, ciprofloxacin and tetracycline. Mutant AK67 with MIC of amikacin 64 mg/L was also resistant to carbapenems (imipenem and meropenem) and fluoroquinolones (norfloxacin and ciprofloxacin), and had slightly elevated MIC of ceftazidime.

Resistance rates of CFPA isolates are generally much higher than those from non-cystic fibrosis patients due to extensive antibiotic use, the adaptive nature and mode of growth of *P. aeruginosa* in the CF-lung [216]. The MICs of amikacin for resistant Swedish cystic fibrosis *P. aeruginosa* (CFPA) isolates were 16 - 256 mg/L (***Table 15***) had higher MICs of other aminoglycosides. Most of these isolates also showed resistance against fluoroquinolones, penems and ceftazidime. The amikacin sensitive CF isolates were susceptible to all antibiotics tested. MICs of tetracycline showed only small variations in mutants and Swedish clinical isolates. Among the Danish CFPA isolates; isolate A1, B2, C2, D2 and D3 were classified as multi-resistant according to the American CF Foundation, that is, resistant to all agents in at least two of the following group of antibiotics: beta-lactams, aminoglycosides and fluoroquinolones [217]. Such isolates were carried by four of the six patients.

The Swedish CFPA isolates were from different patients and there was no cross transmission between patients as determined by pulse field gel electrophoresis (data from the Scandinavian Cystic Fibrosis Study Consortium). Among the Danish CFPA

isolates according to fingerprinting, all but patient CF89 carried the same type of isolate in 1994 and 1997 [197].

4.3.3 Aminoglycoside resistance mechanisms in *P. aeruginosa* laboratory mutants (Paper II)

The amikacin resistant mutants of *P. aeruginosa* could be divided into two groups, based on MIC to amikacin (***Table 14***). The first group (AK66, AK3, AK54, AK38, AK 6, AK4 and AK73) with slightly elevated MIC of amikacin (1 - 2 dilution steps) also had slightly elevated MexY mRNA (8 – 21×PAO1), and in six of these mutants, no significant increase in *mexZ* mRNA expression. The second group, comprised of three mutants (AK14, AK76 and AK67), had high amikacin MICs (64 mg/L) and one mutant (AK67) from this group had the same mutations as a clinical isolate (Cfz09). This mutant had alterations in *mexZ* (deletion of four nucleotides TTCA at position (233-236) and a single nucleotide change (C215→T) in the intergenic region. The other mutants had *mexZ* and the intergenic region between *mexZ* and *mexX* similar to the reference strain PAO1. Moreover AK67 produced higher MexZ and MexY mRNA (>200 fold of that of PAO1) and was more resistant to penems, fluoroquinolones and ceftazidime.

The MICs and mutations found in this isolate indicate a regulation of the *mexXY* operon, probably similar to the regulation of *mexAB-oprM* operon, where MexR regulates the operon negatively, and the operator site for *mexR* and the gene *mexA* are located close to each other (28, 29). The ratio of MexR and MexB mRNA level is closed to one in MexAB-overproducing-*P. aeruginosa* mutants (Personal unpublished data) as was the MexZ/ MexY mRNA ratio in mutant AK67 (***Table 14*****).** Another type of regulation must be present in the nine mutants with no detectable changes in the regulatory regions. Such systems have been described in *E. coli* [51], where a large number of genes for antibiotic resistance are controlled by global regulatory systems. There was a correlation between the production of MexY mRNA and the level of resistance, and mutant AK67, which produced the highest levels of mRNA also showed a multidrug resistance phenotype, in agreement with the findings of Okamoto *et al.* [93].

Table 14. *Summary of MICs (mg/L), mutations in the regulatory gene mexZ and the intergenic region (mexZ-mexX) and expression of mexZ, mexY, oprM and oprD in PAO1 mutants. Sorting order is according to expression of mexY.*

Strain ID	MIC mg/ L							Mutations		mRNA expression				
	AMK	TOB	NET	GEN	MER	IMP	CIP	*mexZ*	*mexZ-mexX*	*mexZ*	*mexY*	*mexB*	*oprM*	*oprD*
PAO1	2	1	2	4	0.25	2	0.125	-	-	1.0	1.0	1.0	1	1.0
AK66	8	2	16	4	2.0	4	0.0625	-	-	10.0	8.2	2.0	0.2	2.02
AK3	8	1	8	4	0.25	2	0.125	-	-	2.0	13	0.5	1.6	0.10
AK54	8	2	8	4	0.25	2	0.125	-	-	1.5	14	0.5	0.6	0.06
AK38	8	1	2	4	0.5	2	0.125	-	-	1.7	14	0.8	0.0	0.06
AK6	4	1	8	8	0.25	4	0.125	-	-	1.7	15	0.3	0.4	0.04
AK4	4	1	4	4	0.5	2	0.125	-	-	1.4	18	0.3	0.8	0.11
AK73	4	1	4	4	0.25	2	0.125	-	-	2.1	21	0.5	0.0	0.08
AK76	64	4	32	8	0.5	2	0.125	-	-	6.0	25	0.6	0.2	0.36
AK14	64	4	32	16	0.125	2	0.25	-	-	3.3	44	0.3	0.2	0.05
AK67	64	4	32	16	4.0	8	2	ΔTTCA(233)	C(215)→T	228.0	280	1.1	0.1	0.24

Abbreviations: AMK, amikacin; TOB, tobramycin; NET, netlimicin; GEN, gentamicin; MER, meropenem; IMP, imipenem; CIP, ciprofloxacin; Δ, deletion .

The MIC breakpoints according to NCCLS for different antibiotics are as follows: CIP:≥ 4 mg/L, S≤1 mg/L; AMK: R≥64 mg/L, S≤16 mg/L;TOB:R≥16 mg/L, S≤4 mg/L GEN: R ≥ 16 mg/L, S≤4 mg/L; NET: R ≥32 mg/L, S≤8; IMP: R ≥ 16 mg/L, S≤4; MER: R ≥ 16 mg/L, S≤4; where R represents the resistant and S represents sensitive.

4.3.4 Aminoglycoside resistance mechanisms in Swedish CFPA isolates (Paper II & IV)

The sequence analysis *mexZ* and intergenic region between *mexZ* and *mexX* of Swedish CFPA isolates rendered different pattern such as single point mutations, frameshift mutations and deletions (***Table 15***). These isolates (MIC amikacin 4 - 256 mg/L) have shown a more complex picture than the mutants, and some of the isolates probably have more than one mechanism of resistance to aminoglycosides. The wide variety of base substitutions, deletions and insertions, found in *mexZ* and intergenic region between *mexZ* and *mexX* could be due to the environment characteristics in the CF lung with a high frequency of hyper-mutable isolates [218, 219]**.** Isolate Cfz09 had the same changes that were found in mutant AK67 (***Table 14***). Among the sensitive isolates, Cfz22 had mutations in both *mexZ* and in the intergenic region between *mexZ* and *mexX* and Cfz13 had an insertion of six nucleotides in *mexZ.* Isolates Cfz04, Cfz07 and Cfz12 had wild type *mexZ* gene but they harboured insertions and point mutations in the intergenic region between *mexZ* and *mexX*, in contrast to isolate Cfz01(MIC of amikacin 64 mg/L), which had wildtype sequence of both mexZ and intergenic region between *mexZ* and *mexX* but still hyper-produced *mexY* mRNA.

It is evident that MexXY plays a role in aminoglycoside resistance in CF isolates, and in some cases also contributes to the multidrug resistance phenotype. MexY mRNA was overproduced (3.4 to 727 time's higher amount of MexY mRNA than PAO1) in all except two of the amikacin resistant isolates (Cfz04 & Cfz08). However, there was no linear relationship between the overexpression of MexY mRNA and the mutations found in the gene for the regulatory protein or intergenic regions. Most of CF isolates with elevated MexY mRNA were also resistant to imipenem and meropenem. One sensitive isolate (Cfz13) with MIC of 4 mg/L also overproduced MexY mRNA (58 × PAO1), and another isolate (Cfz22) had mutations without overproduction of MexY and was susceptible. Possible explanations may be that the cell wall of isolate Cfz13 had increased permeability to antibiotics, probably due to overexpression of *oprD,* which negated the effect of the efflux pump. This isolate was in fact hypersusceptible to imipenem and meropenem. Another possibility is alterations in the pump protein in the region for substrate binding, which may have changed its specificity [153, 220]. For some of the isolates, in particular Cfz08 and Cfz04, which did not overproduce MexY mRNA, other resistance mechanisms must be present.

Table 15. *Minimum inhibitory concentrations, amino acid alterations and relative expression of mRNA from Swedish Pseudomonas aeruginosa isolates*
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CF Isolates	MIC mg /L							Genetic alterations		mRNA Expression			
	AMK	TOB	NET	GEN	MER	IMP	CIP	MexZ	*mexZ-mexX*	*mexY*	PA5471	*mexZ*	*oprM*	*oprD*
Sensitive														
PAO1	2	1	2	4	0.25	2	0.125	-	-	1	1	1	1	1
Cfz21	4	1	4	2	0.125	2	0.125	-	-	0.1	1	2.6	2.7	0.66
Cfz17	2	1	4	2	0.5	2	0.25	-	-	0.4	2	2.4	11	1.9
Cfz18	1	1	1	0.5	0.0625	2	0.5	-	-	0.7	2	1.5	1.6	0.34
Cfz22	2	1	4	2	0.125	0.5	4	Leu(CTG)4→Pro(CCG)	A(42)→G, C(207)→T	1.6	1	4.7	0.2	0.02
Cfz13	4	1	4	2	0.625	0.25	1	Insertion[a]	-	58	1	3.8	1.7	12
Resistant														
Cfz08	128	8	32	32	32	>32	2	Leu(CTC) 105→Arg(CGA)	ΔG(39)	0.1	0.01	1.9	1.7	0.02
Cfz04	32	2	16	8	0.125	4	0.125	-		2.5	0.14	2.3	0.4	0.005
Cfz26	16	2	8	4	4	32	2	Cys(TGC)80→Arg(CGC)	Insertion[b]	3.4	1	7.6	41	0.68
Cfz03	32	4	32	32	32	>32	8	ΔGT(205)	-	36	1	1.3	5.5	0.03
Cfz06	64	4	32	16	>32	>32	4	Glu(GAA)116 →Lys(AAA)	-	55	0.34	0.6	1.3	0.01
Cfz15	64	4	32	16	32	>32	4	Insertion[a]	-	57	1	1.9	7.5	0.33
Cfz10	256	4	128	64	4	>32	4	Arg(CGC)32→Cys(TGC)	-	75	1	2.5	0.6	0.11
Cfz09	16	2	12	8	1	4	2	ΔTTCA(233)	C(215)→T	77	4	7.4	0.2	2.32
Cfz14	128	4	128	32	>32	>32	0.5	Insertion[a]	-	87	2	8.6	1.2	0.58
Cfz07	128	8	>32	32	>32	>32	>32	-	A(178) →G	153	1	4.0	0.2	0.02
Cfz01	64	4	128	32	0.5	4	8	-	-	185	4	0.7	1	0.09
Cfz16	64	2	32	8	>32	>32	8	Leu(CTG)4→Pro(CCG)	-	209	9	4.4	7.1	0.66
Cfz12	128	8	128	64	0.25	0.5	8	-	C(224)→T	258	6	3.1	1.9	0.03
Cfz05	128	8	128	32	0.5	4	0.5	Arg(CGC)159→Pro(CCG)	-	325	2	1.5	0.2	0.22
Cfz02	64	4	64	16	>32	>32	1	Gln(CAG)107→Pro(CCG)	Insertion[c]	727	6	2.1	2.4	0.02

[a]Insertion of 6 nucleotides ACAAGA at nucleotide positions 68-73, original frame restored ; [b]Insertion of G between nucleotides 195 and 196; [c]Insertion of C in between nucleotides 149 and 150

4.3.5 Role of outer membrane proteins in aminoglycoside resistance (Paper II)

The *mexXY* efflux system lacks the coding sequence for outer membrane protein and *oprM* from *mexAB-oprM* system can substitute the function [150]. The expression of this operon is negatively regulated by MexR. In one report MexAB-OprM has been shown to contribute to aminoglycoside resistance in low ionic strength environment [221]. The expression of 1st two genes (*mexA-mexB*) in this operon is growth phase regulated [222] where as the third gene is not [223], which support the suggestions that *oprM* has a second promoter [224]. Most mutants and some CF isolates had OprM mRNA levels that were even lower than PAO1 (***Table 14 & 15***). However four of the Swedish CFPA isolates; Cfz15, Cfz16, Cfz17 and Cfz26 had elevated OprM mRNA production. Two of these isolates (Cfzz15 and Cfz16) which have produced high levels of OprM mRNA, may be due to the overexpression of *mexAB-oprM*. If so, then in these two isolates *mexXY* and *mexAB-oprM* are co-regulated as they expressed high levels of MexY mRNA. However the other two high levels of OprM mRNA producer have expressed very low level of MexY mRNA which is also supported by the findings that, the expression *mexB* in laboratory mutants was close to the level of reference strain PAO1. It can also be that *oprM* has a second regulatory promoter. Some other group proposed that MexXY can also utilize outer-membrane proteins other than OprM, such as OpmG, OpmI and OpmH [93, 155].

Okamoto *et al.* [93] reported that MexAB-OprM is the main efflux system that extrudes carbapenems but that MexXY also extrudes imipenem to a lower extent, and one mutant had increased MICs to carbapenems. Since altered regulation of *oprD* may affect antibiotic susceptibility, we also determined its expression. Among the mutants only AK66 produced higher amount of OprD without any obvious effect on MIC of carbapenems. Mutant AK67 with mRNA for MexY >200 x PAO1 had elevated MIC of carbapenems, but that may be an effect of the MexXY efflux pump. Among the clinical isolates expression OprD mRNA was significantly elevated in isolate Cfz13 (12 x PAO1), and decreased in 11 of the clinical isolates. Isolate Cfz13 with over expression of *oprD* was hypersensitive to meropenem and imipenem. *oprD* downregulation was observed in five isolates with elevated MIC of carbapenem (Cfz08, Cfz03, Cfz06, Cfz07, and Cfz02), but there was no correlation between MIC and amount of OprD mRNA for the remaining isolates.

4.3.6 Aminoglycoside resistance mechanisms in Danish CFPA isolates (Paper IV)

The pattern of changes in *mexZ* gene, which was observed in Danish CFPA isolates were different from Swedish CF isolates, they followed a specific pattern of changes which has not been observed in Swedish isolates.

Table 16. *Minimum inhibitory concentrations, amino acid alterations and relative expression of mRNA for Pseudomonas aeruginosa isolates from six CF patients in 1994 and 1997 from Danish Cystic Fibrosis Centre*

Patients	Isolates	MIC (mg/ L)			Amino acid alterations	mRNA Expression	
		AMK	TOB	CIP	MexZ	*mexY*	PA5471
	PAO1	2	0.5	0.12	-	1	1
CF166	A1 (1994)	>256	8	1	Frameshift*	15	1
	A2 (1997)	32	2	4	Frameshift*	12	1
	A3 (1997)	128	4	1	Frameshift*	51	2
CF222	B1 (1994)	2	4	2	L205P	61	2
	B2 (1994)	>256	12	2	L205P	137	4
	B3 (1997)	>256	1	8	L205P	79	1
	B4 (1997)	64	4	0.5	Frameshift*	111	2
CF86	C1 (1994)	16	2	2	Frameshift*	3	1
	C2 (1994)	128	128	2	Frameshift*	334	1
	C3 (1997)	128	6	8	Frameshift*	48	<1 (0.60)
	C4 (1997)	64	4	2	Frameshift*	75	1
CF59	D1 (1994)	24	2	2	L205P	3	5
	D2 (1997)	>256	>256	4	L205P	255	1
	D3 (1997)	>256	8	0.5	Frameshift*	278	1
CF21	E1 (1994)	16	2	0.5	Deletion**	104	2
	E2 (1994)	32	1	1	Deletion**	162	2
	E3 (1997)	32	2	4	Frameshift*	235	<1 (0.80)
	E4 (1997)	32	2	1	Deletion**	78	2
CF89	F (1994)	20	2	0.5	Frameshift*	41	<1 (0.70)
	G (1997)	8	1	2	A47V	1	3

Abbreviations: CIP, ciprofloxacin; AMK, amikacin; TOB, tobramycin; Amino acids: L; leucine, P; Proline, A; Alanine, V; Valine

The MIC breakpoints according to NCCLS for different antibiotics are as follows: CIP:≥ 4 mg/L, S≤1 mg/L; AMK: R≥32 mg/L, S≤16 mg/L and TOB:R≥8 mg/L, S≤4 mg/L where R represents the resistant and S represents sensitive.

*Insertion of 16 nucleotides (GCGGGCGCGGCGAACT) downstream from position 450, results in frameshift beyond the Arg codon 153

**57 amino acids have been deleted from positions 135-192

In MexZ of isolates E1, E2 and E4; 57 amino acids have been deleted from positions 135-192 and interestingly, the original reading frame is maintained. In these 3 strains the hyperproduction of MexY mRNA correlates the inability of MexZ to stop transcription, which gives rise to 80 to 150 fold increased expression of *mexY* compared to the wild type strain PAO1 (***Table 16***). All these 3 isolates were from patient CF21, two (E1 and E2) from 1994 and E4 from 1997 were identical by ribotyping [197]. Eleven out of 20 CF isolates had an insertion of 16 nucleotides (GCGGGCGCGGCGAACT) downstream from position 450 which results in frameshift beyond the arginine codon 153, and the amino acid frame was not restored. In 3 patients (CF222, CF59 and CF21) this frameshift mutation was found in strains isolated on 1997. All *P. aeruginosa* strains isolated from CF86 and CF166 in 1994 as well as in 1997 carried the frameshift mutation. Effect of this frameshift mutation on MexY mRNA overproduction was prominent in all strains except C1 which produced only 3 fold MexY mRNA compared to the control strain PAO1. In all these strains the MICs of amikacin were significantly higher, ranging from 16 to >256 mg/L. Among the CFPA isolates without any frameshift or deletion mutation, strains B1, B2, B3, D1 and D2 had a single nucleotide change, $T_{614}\rightarrow C$ which resulted in $Leu_{205}\rightarrow Pro$ substitution at the C terminal end of MexZ. The significance of this point mutation is unknown but a similar mutation has been reported by Vogne *et al.* in a CF strain (2117R) [148]. Strain D1 (from 1994) from patient CF59 failed to produce significantly elevated levels of MexY mRNA and had MIC of amikacin of only 24 mg/L. As strains D1, D2 and D3 were similar by PFGE, strain D2 (from 1997) had the same $Leu_{205}\rightarrow Pro$ mutation with much elevated production of MexY mRNA and MIC of amikacin >256 mg/L, probably ruling out the effect of this mutation on the regulation of MexXY efflux system. On the contrary, in strain G change of nucleotide $C_{140}\rightarrow T$ resulted in point mutation $A_{47}\rightarrow V$ in the N-terminal end of MexZ, but this strain had wild type MexY mRNA level with much less elevated MIC of amikacin compared to other strains. This isolate was dissimilar to its 1994 counterpart strain F, which might be due to the fact that patient in CF89 strain G had replaced strain F over the period of 3 years.

The occurrences of these different mutations in *mexZ* from CF patients are common [225] which results in significant hyperexpression of *mexY* although there is no simple correlation with MIC levels. Interestingly all the Danish CFPA isolates had wild type mexZ-mexX intergenic region compared to Swedish isolates.

4.3.7 Role of PA5471 gene product in modulation of MexZ function

It has been recently reported that the gene PA5471 is inducible by the similar ribosome targeting drugs that induce MexZ regulated MexXY operon and it plays a role in MexXY mediated antimicrobial resistance [226]. Mutant strains with non-functional PA5471 gene have been shown to be compromised for ribosome targeting drug inducible MexXY expression and MexXY-OprM mediated antimicrobial resistance [226]. The mRNA expression of PA5471 gene has been measured in this study by realtime PCR. In all Danish CFPA isolates with *mexZ* deletions (E1, E2 and E4), the level of PA5471 expression was increased twofold compared to the wild type. The

frameshift mutants had also shown the similar levels of PA5471 as the deletion mutants except strains C3, E3 and F in which the expression of PA5471 seemed to be downregulated (***Table 16***). These 3 strains produced significantly elevated levels of *mexY* mRNA and this down regulation of PA5471 did not affect their MICs of amikacin. The effect of these deletion and frameshift mutations probably resulted in unstable dimer formation and/or binding of MexZ to its target site. On the contrary, 3 to 5 fold upregulation of PA5471 was found in strains D1 and G but these strains failed to produce significant amounts of *mexY* mRNA concordant with their MICs of amikacin; thus, other mechanisms were involved in these two strains. Strain D which is the highest producer of PA5471 mRNA produced only 3 fold MexY mRNA and it was moderately resistant to tested aminoglycoside which indicates that in CF strains the regulation of MexY expression is a complex process and involves more regulatory loci in the *Pseudomonas* chromosome.

Among the Swedish CFPA isolates 4 to 9 fold increase in the PA5471 mRNA expression resulted in significant increase in mexY mRNA expression (***Table 15***). Isolate Cfz16 produced highest level of PA5471 mRNA (9 x PAO1) with a point mutation near the 5′ end (L_4→P) followed by 200 fold increase in *mexY* mRNA level. In comparison, isolate Cfz22 which produced PA5471 mRNA similar to the control strain and had similar changes in *mexZ* and a point mutation in the intergenic region between *mexZ* and *mexX,* expressed only a slightly elevated level of *mexY* mRNA (***Table 15***). More interestingly, isolate Cfz09 with a deletion in *mexZ* and a point mutation in the intergenic region produced less than half amount of *mexY* mRNA compared to strain Cfz01 with wild type *mexZ* and intergenic region between *mexZ* and *mexX*. The expression levels of PA5471 mRNA were similar (4 fold) in these two isolates. Isolate Cfz02, which was the highest level of *mexY* mRNA producer, also produced 6 times higher PA5471 mRNA, similar to Cfz12.

These observations are also in notion with the findings of Morita *et al*.[226] suggest that combination of changes in *mexZ* and elevated level PA5471 gene product can give rise to higher expression of MexY and thereby can contribute to increase the MICs to aminoglycoside in CFPA isolates.

4.3.8 Aminoglycoside modifying enzymes in CFPA isolates (Paper II & IV)

Commonly reported genes for aminoglycoside modifying enzymes [227] were not found any of the CFPA isolates investigated. Amikacin is less susceptible to modifying enzymes than other aminoglycosides [120] although one 3′-phosphotransferase with high activity against amikacin has been described [201]. The Swedish CFPA isolates as well as the Danish were negative for the presence plasmid borne aminoglycoside-modifying genes *aac(6′)-Ib* and ant(4′)-*IIb* or chromosomal *aph(3′)-IIps* gene which mainly confers amikacin resistance. The drug modifying enzymes are encoded by genes that are often associated with transmissibility among organisms [161] and in very few cases enzymatic mechanisms of aminoglycoside resistances is combined with impermeability type resistance (efflux mediated resistance mechanism). Another report

suggests [166] that non CF *P. aeruginosa* isolates are in most cases resistant by the presence of aminoglycoside modifying enzymes.

4.3.9 Non-enzymatic mechanisms of aminoglycoside resistance in CFPA isolates (Paper IV)

P. aeruginosa can account for gradual increase toward higher MICs of aminoglycosides through non enzymatic mechanisms other than efflux pump or aminoglycoside modifying enzymes. Recently, it has been shown that mutants of strain PAO1 with Tn5-Hg insertions in any of the chromosomal genes *nuoG*, *galU*, *mexZ* or *rplY* have shown a two fold increase in MICs of aminoglycosides [158]. In *P. aeruginosa* strains in cystic fibrosis lung, accumulation of these types of chromosomal changes might contribute to gradual increase in MICs of aminoglycosides. In paper IV, the entire *galU* and *rplY* gene were sequenced from all isolates for the presence of unexpected stop codon or any other kind of changes that lead to production of altered proteins. The NADH dehydrogenase I chain G gene *nuoG* belongs to an operon *nuoABDEFGHIJKLMN* consisting of 13 consecutive genes and presence of stop codon in any of these gene will lead to the production of truncated mRNA. To confer that the operon is transcribed completely we have qualitatively analysed the mRNA expression of two genes *nuoH* and *nuoN* by melting curve analysis of realtime PCR method.

Both the Danish and Swedish Cystic fibrosis *P. aeruginosa* isolates were found to have wild type amino acid sequence for *rplY* and *galU*. On the other hand only one (Cfz02) out of 40 *P. aeruginosa* isolates tested, found to has disrupted *nuoABDEFGHIJKLMN* operon according to realtime PCR analysis. This isolate produced 727 fold of *mexY* mRNA compared to the reference strain and had changes both in mexZ and the intergenic region between *mexZ* and *mexX*.

4.3.10 Ribosomal A site mutation (Paper IV)

Aminoglycoside antibiotics target the A site located at the 3´ end of the 16 rRNA and eventually lead to the misreading of the genetic code. Point mutations in this region contribute to resistance to aminoglycoside antibiotics in *Escherichia coli*, and possibly also in *P. aeruginosa*. The ribosomal A site of *P. aeruginosa* consists of nucleotide 1400 and 1408 to 1489 to 1500 (*E. coli* numbering system). Among these nucleotides, a single A1408→ G confers amikacin and other 2-deoxy streptamine resistance [182]. Moreover, mutations in any of the uracil residues forming U1406-U1495 pair or double mutation of both of the nucleotides confer high level of aminoglycoside resistance [181, 228]. As *P. aeruginosa* has 4 copies of 16S rDNA and presence of any A site mutation in single or multiple 16s rDNA copy could result in higher MICs respectively. All the copies of 16S rDNA are flanked by tRNA-Ile (http://V2.pseudomonas.com) gene which makes it difficult to differentiate between each copy of 16S rRNA genes. Among total of 40 CFPA isolates we have analysed 5 Danish and 8 Swedish isolates for the presence of A site mutation by pyrosequencing. All the strains had wild type

16S rRNA gene sequence in all four genes. Thus, mutations in genes for 16S rRNA may be uncommon in aminoglycoside resistant *P. aeruginosa* strains.

In conclusion, it was found that the MICs of clinical strains of *P. aeruginosa* isolated from CF patients in 1997 was increased at least twofold compared to the strains isolated from 1994. These increases in MIC value reflect that the long term use of antibiotics not only selects strains for the increase of specific resistance but also for resistance to other antibiotics with different structures and chemical properties. In the *P. aeruginosa* isolates tested in this study, MexXY-OprM efflux system mediated aminoglycoside resistance was found as the most common mechanism of resistance in cystic fibrosis patients and the regulatory gene *mexZ* was mostly altered in both Danish and Swedish cystic fibrosis *P. aeruginosa* isolates.

5 CONCLUSION

The study focused on the chromosomally mediated antibiotic resistance in *N. gonorrhoeae* and *P. aeruginosa*.

We have concluded that alteration in GyrA subunit of DNA gyrase is the main determinant of fluoroquinolone resistance in *N. gonorrhoeae*. Our study suggests that introduction of additional mutations in *gyrA* and/or *parE* as well as alterations of *porB1b* contribute to ciprofloxacin resistance.

Downregulation of OprD has been found to be the main mechanism of carbapenem (imipenem) resistance. Sequencing of penicillin binding protein genes coding for PBP1b, PBP2, PBP3 and PBP6 showed no differences in amino acid sequence in clinical strains and in transconjugants analyzed in this study. In cystic fibrosis patients who become chronically colonized with *P. aeruginosa* the same strains persists in their lungs. The risk of acquiring foreign DNA molecules or antibiotic resistant plasmid is relatively low in CF respiratory environment which is also supported by the fact the *P. aeruginosa* is very resistant to foreign DNA. In most cases progenies with different phenotypes of the same strain perpetuate in the lung. All these mentioned factors allowed us to focus more on changes in chromosomal determinants which contribute to aminoglycoside resistance in CF *P. aeruginosa* isolates. The major chromosomal changes we have found in our study are in the regulatory genes for the efflux pump MexXY followed by the over expression of pump protein MexY as the dominating mechanism of aminoglycoside resistance in CF *P. aeruginosa* isolates.

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