Quinolones were introduced into clinical use in 1962 in the form of nalidixic acid, a fully synthetic agent with bactericidal effects on most Enterobacteriaceae at clinical concentrations (Van Bambeke et al 2005). Nalidixic acid is the parent compound of the quinolone class of antibiotics. Quinolones available for clinical use have been classified into four generations, mainly on the basis of their spectrum of activity (Ball, 2000). Following the lead of flumequine, the second generation of quinolones had the major feature of a fluorine substituent (F) at position 6 (hence the name of fluoroquinolones often given to the whole class), which increased activity markedly.

Figure 2.1 Structure active relationships of Quinolones
(Figure is taken from Van Bambeke et al 2005)
Figure 2.2 Pharmacophore and structures of the main quinolones that have been approved for human use

In the Figure 2.2, the names in bold refer to compounds in large-scale clinical use in Europe. Names in italic refer to compounds for which commercialisation has been suspended or severely reduced because of side-effects and/or a decision of their registration holders (Table is taken from Van Bambeke et al 2005).
2.1 Epidemiology of \textit{qnrA}

After the initial discovery of \textit{qnrA} in a \textit{K. pneumoniae} isolate obtained in 1994 from the urine of a patient in Alabama, USA, efforts were made to find this gene elsewhere. A survey for \textit{qnrA} by PCR of more than 350 Gram-negative isolates collected mainly in the 1990s and chosen to include a broad geographic range and a variety of genera of Gram-negative bacteria found \textit{qnrA} in only six isolates (four \textit{E. coli} and two \textit{Klebsiella spp}), all from the same centre in Alabama where the original strain had been detected, and all collected between July and December, 2004 (Jacoby et al 2003). All six isolates transferred nalidixic acid resistance together with a gene encoding FOX-5 β-lactamase, which was also present on pMG252. Strikingly, while FOX-5 β-lactamase-carrying isolates were still present in surveys of 1995 and 2001 organisms from the same centre, \textit{qnrA} was no longer found (Jacoby et al 2003). Since this early study, about 20 more epidemiological surveys have been reported (Figure 2.3). Most have used PCR methodologies to examine clinical \textit{Enterobacteriaceae} collected in the late 1990s or early 2000s for \textit{qnrA}. Although the gene seems to be uncommon in general populations of Gram-negative isolates, prevalence in certain organisms carrying extended spectrum β-lactamases has exceeded 20%. In these studies, \textit{qnrA} was found in all populated continents except South America, and in most clinically common \textit{Enterobacteriaceae}. These species include \textit{E. coli}, \textit{Klebsiella spp} (\textit{K. pneumoniae} and \textit{Klebsiella oxytoca}), \textit{Enterobacter spp} (\textit{Enterobacter cloacae}, \textit{Enterobacter amnigenus}, and \textit{Enterobacter sakazakii}), \textit{Citrobacter freundii}, and \textit{Providencia stuartii} (Robicsek et al 2006).
2.2 Mode of Action and Mechanisms of Resistance

2.2.1 Interaction with Bacterial Type II Topoisomerases.

Several mechanisms have evolved in bacteria which confer antibiotic resistance. These mechanisms can chemically modify the antibiotic, render it inactive through physical removal from the cell (efflux pumps), or modify target site so that it is not recognized by the antibiotic (Todar, 2013).

Fluoroquinolones are the only class of antimicrobial agents in clinical use that are direct inhibitors of bacterial DNA synthesis. Fluoroquinolones inhibit two bacterial enzymes, DNA gyrase and topoisomerase IV, which have essential and distinct roles in DNA replication. The quinolones bind to the complex of each of these enzymes with DNA; the resulting topoisomerase-quinolone-DNA ternary complex subsequently leads to the generation of double-stranded breaks in DNA and blocks progress of the DNA replication enzyme complex (Figure 2.4). Ultimately, this action results in damage to bacterial DNA and bacterial cell death (Dalhoff, 2012).
Resistance to quinolones occurs by mutation in chromosomal genes that encode the subunits of DNA-gyrase and topoisomerase IV (altered target mechanism), and that regulate the expression of cytoplasmic membrane efflux pumps or proteins that constitute outer membrane diffusion channels (altered permeation mechanism) (Dalhoff, 2012). The intracellular concentration of the accumulated antibiotic within the bacterial cell is effluxed out by the help of efflux pumps (Figure 2.4).

**2.2.2 Plasmid Mediated Fluoroquinolone Resistance.**

The genetic information for target site or efflux resistance mechanisms is commonly chromosomally encoded. However, the emergence of plasmid-mediated and thus
transferable fluoroquinolone resistance has also been reported; several mechanisms are known:

1. Qnr,
2. Aminoglycoside acetyltransferase *aac(6')-Ib-cr*,
3. Efflux Pumps - OqxAB, QepA

The emergence of plasmid-mediated quinolone resistance was first found in strains of *K. pneumoniae* in one region of the United States in 1998 (Martínez-Martínez et al 1998) and shown to be due to a member of the pentapeptide repeat (PPR) family of proteins *Qnr* (later named QnrA). In the following years, several distantly related plasmid mediated *Qnr* determinants were described in *Enterobacteriaceae* (*qnrB, qnrC, qnrD, qnrS*) (Jacoby et al 2008). They have been identified worldwide and are almost always associated with the production of expanded spectrum β-lactamases (Robicsek et al 2006).

*Qnr* interacts with DNA-gyrase and topoisomerase IV to prevent quinolone inhibition (Nordmann & Poirel, 2005; Tran & Jacoby, 2002). *Qnr* protein causes nalidixic acid resistance and reduced susceptibility to or low-level fluoroquinolone resistance (Nordmann & Poirel, 2005). The *qnr*-genes have been found in ciprofloxacin-susceptible isolates as well as quinolone resistant isolates, suggesting that their presence promotes higher level resistance due to chromosomal mutation, as has been shown in the laboratory. Therefore, the presence of *qnr* genes in clinically relevant species of both, Gram-positive and Gram-negative bacteria may foster quinolone resistance development. Furthermore, *qnrA* and *qnrB* genes are usually integrated into integrons which harbor other antibiotic resistance genes such as β-lactamases or aminoglycoside inactivating enzymes. Although *qnrS* genes are not harbored by integrons, they are associated with transposons containing TEM-1 type β-lactamases (Hernandez et al 2011).

Another plasmid-encoded quinolone resistance determinant was identified, a variant of the *aac(6')-Ib* gene encoding an aminoglycoside acetyltransferase. The bifunctional aminoglycoside and fluoroquinolone active variant *aac(6')-Ib-cr* catalyzes acetylation of both drug classes. The variant enzyme has acquired the ability to acetylate ciprofloxacin and norfloxacin and reduces ciprofloxacin’s activity four fold. The gene *aac(6')-Ib-cr* may be more widespread than *Qnr*-determinants. Both, *Qnr* and *aac(6')-Ib-cr*-production are associated with the ESβL production, thus, representing a second mechanism of co-selection of drug-resistance due to exposure to chemically unrelated agents.
2.3 Role of efflux pumps in antibiotic resistance

Efflux pumps contribute to multidrug resistance as they expel different types of antibiotics and chemicals such as dyes, organic solvents, detergents, molecules needed for the cell-cell communication, biocides, and metabolic products. Hence understanding the mechanisms by which these pumps act and how to overcome its activity opens the door for restoring the antibiotic activity and constitute a promising target for novel antibacterial agents (Piddock, 2006; Askoura et al 2011).

Drug efflux is a key mechanism of resistance in Gram negative bacteria. These systems pump solutes out of the cell. Efflux pumps allow the microorganisms to regulate their internal environment by removing toxic substances, including antimicrobial agents, metabolites and quorum sensing signal molecules (Pearson et al 1999).

Efflux pumps may be formed by a single-component or by multiple components, with the latter being found exclusively in Gram negative bacteria. Bacterial drug efflux pumps have been classified into six families by the number of components, the number of transmembrane- spanning regions, the energy source used by the pump and the types of molecules that the pump exports (Piddock, 2006) (Figure 2.5).

1. The ATP-binding cassette (ABC) superfamily; (Lubelski et al 2007)
2. The major facilitator superfamily (MFS); (Pao et al 1998)
3. The multidrug and toxic compound extrusion (MATE); (Kuroda & Tsuchiya, 2009)
4. The small multidrug resistance (SMR) family; (Jack et al 2001)
5. The resistance-nodulation-division (RND) superfamily; (Li & Nikaido 2004)
6. The drug metabolite transporter (DMT) superfamily (Piddock, 2006).

Those six classes obtain energy required for the active transporting either from H+ protons (RND, SMR, and MFS), Na+ dependant (MATE), or by hydrolysis ATP (ABC). (Piddock, 2006 ; Misra & Bavro, 2009).

The major clinically relevant efflux systems in Gram-negative bacteria belong to the RND superfamily and are typically composed of a cytoplasmic membrane pump, a periplasmic protein and an outer membrane protein channel. Over the past several years, while further characterizing previously-studied drug efflux pumps including RND systems, novel efflux systems have also been identified in Gram-negative bacteria (Li & Nikaido, 2009). In spite of the studies related to the importance of efflux pumps in biofilm growth and their relevance in biofilm antimicrobial resistance, the exact role of these efflux systems has yet to be determined.
2.4 The Relationship between Quorum Sensing and Efflux Systems

Quorum sensing (QS), or cell-to-cell signalling, is the controlled expression of specific genes in response to extracellular chemical signals produced by bacteria themselves (Pearson et al 1999). It is well known that QS plays a role in the development of biofilm (Davies et al 1998). The connection between QS and biofilm is known as sociomicrobiology (Parsek & Greenberg, 2005). Efflux systems have been implicated in QS regulation, and it is well known that QS controls the expression of a number of virulence factors as well as biofilm differentiation (Chan & Chua 2005; De Kievit et al 2001) demonstrated that QS controlled processes such as biofilm formation were dependent on BpeAB-OprB efflux pump function in Burkholderia pseudomallei. In Pseudomonas aeruginosa, it has been observed that a mutation in a probable RND-like efflux pump transporter down regulates QS-dependent lecA::lux expression (Diggle et al 2002). Studies have also demonstrated that QS is partly dependent upon efflux. Thus, the signal molecule 3OC12-HSL requires active transport through an efflux pump to diffuse across the cell membrane in P. aeruginosa (Pearson et al 1999). Therefore, an increase in efflux pump activity could have several effects on biofilm formation through an increase in the extrusion or intrusion of QS molecules (Hocquet D et al 2007).
2.5 Natural Roles of MDR Pumps - Bile Tolerance of Enteric Bacteria

It has long been considered that the natural physiological role for MDR efflux pumps in bacteria is in the export of noxious substances from the bacterial cell thereby allowing survival in a hostile environment. Efflux pumps predate the antibiotic era, so their natural role is unlikely to be related to antibiotic use; i.e., antibiotics such as fluoroquinolones are unlikely to have selected for pump evolution. The natural environment of enteric pathogens is rich in bile salts and fatty acids, suggesting that one of the many physiological functions of active efflux systems is both the secretion of intracellular metabolites and protection against a variety of substances in this environment. It has been shown for *E. coli*, *S. enterica serovar Typhimurium*, and *Campylobacter jejuni* that mutants lacking components of the AcrAB-TolC pump or the CmeABC pump are hypersusceptible to bile and bile salts (natural antimicrobial substances produced in the avian and mammalian gut as an antimicrobial defence to bacterial challenge) and that mutants that overexpress components of these pumps are resistant to high concentrations of bile and bile salts. Therefore, it has been suggested that the primary function of the *E. coli* AcrAB-TolC and *Campylobacter jejuni* CmeABC efflux pumps of these organisms is to allow enteric bacteria to survive in the presence of bile. Induction of the *E. coli* AcrAB pump is by exposure to bile salts gives rise to MDR (Piddock, 2006).

2.6 Efflux Pumps mediated fluoroquinolones resistance

A third type of plasmid-mediated quinolone resistance has been identified, they are the quinolone efflux pumps OqxAB and Qep, (Perichon et al 2007; Strahilevitz et al 2009). The OqxAB and QepA-proteins confer resistance to hydrophilic fluoroquinolones like norfloxacin, ciprofloxacin, and enrofloxacin, causing a 32- to 64-fold increase in MICs (Perichon et al 2007; Hansen et al 2007). QepA extrudes in addition to quinolones a narrow range of agents such as erythromycin, ethidium bromide, and acriflavine; OqxAB exports a wider range of agents like ethidium bromide, tetracyclines, chloramphenicol, trimethoprim, olaquindox, and the desinfectants like triclosan (Hernandez et al 2011; Hansen et al 2007; Hansen et al 2004).

The *qepA* gene and an aminoglycoside ribosome methyltransferase are part of a transposable element (Yamane et al 2007), so that there is a potential of selection of *qepA* determinants by aminoglycosides and vice versa aminoglycoside resistance by quinolones; the same holds true for *aac(6’)-Ib* gene mediated resistances. Extrusion of chemically
unrelated agents by efflux-pumps represents a third mechanism of cross-resistance. Fluoroquinolone resistance can emerge even in the absence of exposure to this drug class as several co-selection mechanisms favour the emergence of quinolone resistance.

Additional, unknown mechanisms of quinolone resistance must exist as known chromosomally-and plasmid mediated resistance mechanisms plus the presence of the multidrug efflux pump AcrAB were detected in just 50–70% of high-level quinolone resistant *E. coli* clinical isolates with MICs up to 1,500-fold higher than expected (Morgan-Linnell et al 2009). However, a varying degree of efflux pump activity also plays a role in establishing the level of drug resistance/susceptibility in clinical isolates (Aathithan & French, 2011). Therefore, it would be a challenge to develop novel efflux-pump inhibitors (EPIs) which might be used in combination to overcome resistance to the existing antimicrobial agents. Efforts are going onto identify and isolate secondary metabolites from plants as prospective modulators of bacterial resistance (Stavri et al 2007).

### 2.7 The efflux pump inhibitors as new therapeutic agents

The continuous increase in the development of multidrug resistance by many pathogens has resulted in difficulties fighting many infectious diseases. In view of the fact that the majority of those multidrug resistant pathogens express and overproduce efflux pumps that are responsible for the expelling and extruding of the antibiotics from inside the cells, the new direction for other chemotherapeutics is the use of efflux pump inhibitors (EPIs) (Pages & Amaral 2009). Using the (EPIs) together with antibiotics can reduce the invasiveness of *Pseudomonas aeruginosa* besides its role in lowering the antibiotic minimal inhibitory concentration (Hirakata Y et al 2009). For example, the sensitivity to ciprofloxacin by *Pseudomonas aeruginosa* is largely increased upon using this inhibitor proving that efflux pumps play a role in the resistance of this organism to this antibiotic (Tohidpour et al 2009). Thus the inhibition of the efflux pumps is promising in order to

1. Increase the intracellular drug concentration,
2. Restore the drug activity against the resistant strains, and
3. Minimizes further development of resistant strains.

However, this requires the understanding of the structural and physiological mechanisms of the responsible efflux pumps.
The inhibition of efflux pumps can be achieved by different mechanisms as follows:

1. Interference with the regulatory steps needed for the expression of the efflux pump,
2. Chemical changes in the antibiotic structure hence hindering its attachment as the specific substrate,
3. Disruption of the assembly of the efflux pump-components,
4. Inhibition of the substrate (antibiotic) binding by either competitive or non-competitive binding using other compounds,
5. Blocking the outermost pores responsible for the efflux of antibiotic compound,
6. Interference with the energy required for the pump activity (Pages & Amaral, 2009).

Many compounds have been tested for their efflux pump inhibition ability including some analogues for antibiotic substrates and other chemical compounds, but few are used that take into consideration the structure-activity relationship and the spectrum of the activity. The general methodology used for testing the efficacy of these efflux inhibitors is simply performed by comparing the intracellular concentration of the added antibiotic to the bacterial cell culture before and after the addition of the (EPIs) such as phenylalanine arginyl β-naphthylamide (PAβN). If the compound under testing showed higher intracellular concentration of the antibiotic, it is considered as good efflux inhibitor and vice versa (Askoura et al 2011).

2.8 Peptidomimetic compounds (PAβN) as efflux inhibitors against Pseudomonas infections

The most widely used compounds as (EPIs) for Pseudomonas overexpressing MexAB pump are the group of peptidomimetic molecules with phenylalanine arginyl β-naphthylamide (PAβN) as a leading compound (MC-207,110) (Figure 2.6) and other many derivatives with similar structures. The mechanism of action of these inhibitors is through competitive inhibition mechanism, where the efflux pumps recognize them as a substrate instead of the target antibiotics (quinolones mainly ciprofloxacin and levofloxacin) and as long as the pumps expel these inhibitors outside the cells, the antibiotic remains intracellular and increasing in concentration. PaβN, which has a differential behaviour, i.e., it can compete with certain antibiotics and not the other depending on the nature of the efflux pump and the large substrate-binding site. It was also shown that PAβN can restore the activity of other unrelated antibiotics such as chloramphenicol and macrolides; hence,
it can be considered a broad spectrum efflux pump inhibitor. Other derivatives have been designed from PAβN such as MC-04,124 (Figure 2.6), which is more stable in biological fluids, shows less toxicity levels and more activity against *Pseudomonas aeruginosa* overexpressing efflux pumps.

![PAβN molecule](image)

*Figure 2.6 Efflux pump inhibitors (EPIs) - phenylalanine arginyl β-naphthylamide, PAβN Molecular Weight 290.36* (Askoura et al 2011)

The PAβN-derived (EPIs) still remain the most studied and developed family against *Pseudomonas aeruginosa*, though more studies concerning the structure activity relationship, pharmacokinetics, and stability in biological fluids are required. The main advantage of using the PAβN-derived (EPIs) is the difficulty to develop resistance to them, where any pump mutation leading to inhibitor resistance will lead to resistance to the antibiotic substrate. The disadvantage of those compounds is their low affinity to the target that necessitates the use of higher doses and for a longer time in addition to only substrates that share the inhibitor binding site will be affected (Poole K, 2006).

The main drawbacks associated with these EPI compounds is their toxic properties hindering their clinical applications; however, they are used in order to evaluate the different efflux mechanisms expressed by different pathogenic bacteria besides the measurement of the affinity of efflux pumps to them in relation to the antibiotics (Lomovskaya O, 2006). It has been demonstrated that PAβN is more potent as an efflux inhibitor against MexAB-OprM pump of *Pseudomonas aeruginosa* when compared to quinoline derivatives (another class of EPIs) that could be attributed to the difference in the screening protocols for the antibiotic used as a substrate (levofloxacin versus chloramphenicol) for the two EPI classes (peptidomimetics or quinoline derivatives) (Mahamoud A et al 2006).
2.9 The efflux pump energy: targeting the driving force of the mechanism

Compounds that seriously affect the energy level of the bacterial membrane such as carbonyl cyanide m-chlorophenylhydrazone (CCCP) (Figure 2.7), are used in the laboratory to abolish totally the efflux of various molecules (Pages J-M et al 2005). These compounds reduce the viability of the bacterium and cause cell death via the dissipation of the proton-motive force of the membrane. Consequently, there is always the question of whether it is their effect on the efflux pump that is the cause of an increase in the penetration of the antibiotic, or whether it is due to the alteration of the cell envelope itself that results in the death of the bacterium. In addition, some of them, like CCCP, are recognized as highly noxious and cytotoxic and are also substrates of bacterial efflux pumps. Today, no molecule belonging to the energy-blocker family has been developed for clinical use or has been patented (Mahamoud A et al 2007).

![Figure 2.7 Efflux pump inhibitors (EPIs) Carbonyl cyanide m-chlorophenylhydrazone (CCCP), Molecular Weight 204.62(Sigma)](image)

2.10 Quinoline derivatives

This novel class of compounds was discovered by using several screening procedures with Enterobacter aerogenes strains (Mahamoud A et al 2006). These compounds have been assayed for their activity against various MDR clinical strains overexpressing efflux pumps that expel different antibiotics (e.g. chloramphenicol, norfloxacin). Active quinoline derivatives have then been evaluated for their ability to restore the activity of various
antibiotic families (e.g. quinolone, phenicol, cycline) (Mahamoud A et al 2006). Data from many sources confirm the potential of these EPI compounds, and several quinoline derivatives are now considered as broad-spectrum EPIs for rendering antibiotic-resistant *E. aerogenes* and *K. pneumoniae* susceptible to chloramphenicol, tetracycline and norfloxacin. Moreover, the direct action of this family of molecules on the drug extrusion mechanism has been clearly demonstrated by measuring the intracellular concentrations of antibiotics (norfloxacin, chloramphenicol) after their addition to bacterial cultures; their ability to increase the accumulation of the antibiotic has been compared to that resulting from the addition of CCCP or PAβN to the culture (Mahamoud A et al 2006).

2.11 Screening for EPIs

It has been known for many years that EPI exhibit synergy with antibiotics and this has been used to screen for the EPI. The checkerboard assay (Stefanovic Olgica et al 2011) has been used to identify such agents and its variants have been applied to identify potential inhibitors of efflux pumps. Antibiotic accumulation studies have been used to identify potential EPIs. Various substrates that have also been used in accumulation studies include ethidium bromide, norfloxacin, berberine and novobiocin. Assays can be performed in a number of ways to determine the effect of a potential efflux inhibitor on a bacterial strain possessing an efflux pump. One method is the incorporation of an efflux inhibitor midway through a time-course assay in order to detect a difference in fluorescence. Another method is to run two separate time-course assays, one in the absence and one in the presence of an inhibitor to determine any effect a test compound may have as a potential inhibitor. An increase in drug accumulation only in the presence of an inhibitor indicates that the inhibitor is a blocker of an efflux mechanism (Stavri et al 2007).

Based on a literature survey, plants that were known to have antimicrobial activity and that were used in Ayurveda to treat urinary infections were collected. Traditional healers prepare a wide range of healing juices, crude extracts, paste and tincture from various herbs extract. It is possible that there are such potentiating molecules present in the traditional preparations and our project is to identify such potentiating molecules of the major active ingredient. In many cases, the medicinal plant extracts which showed promising potentiation activity with commonly used antibiotics were used as a screening method. We propose to investigate whether such potentiating molecules are present in the traditional medicine preparation.
2.12 Biofilm

Bacteria have traditionally been regarded as individual organisms growing in homogeneous planktonic populations. However, bacteria in natural environments usually form communities of surface-adherent organisms embedded in an extracellular matrix, called biofilms. Current antimicrobial strategies often fail to control bacteria in the biofilm mode of growth. Treatment failure is particularly frequent in association with intracorporeal or transcutaneous medical devices and compromised host immunity. The rising prevalence of these risk factors over the last decades has paralleled the increase in biofilm infections. Biofilm characteristics are described with a focus on new diagnostic and therapeutic targets (Fux et al 2003).

2.13 What is a Biofilm?

Biofilms have been defined as complex microbial associations anchored to abiotic or biotic surfaces. This structure may be formed by a single or multiple microbial species. The cells are embedded in extracellular matrix produced by the biofilms themselves by which they interact with each other and the environment. However, a new definition of biofilm has been proposed taking other physiological attributes of the microorganisms forming biofilm into account. Therefore, biofilm is defined as a microbiologically derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or each other, are embedded in a matrix of extracellular polymeric substances that they have produced and exhibit an altered phenotype with respect to growth rate and gene transcription (Donlan & Costerton, 2002). Biofilm formation has been observed by most of the bacteria found in natural, clinical and industrial settings. The matrix contains several substances such as polysaccharides, proteins and DNA from the microorganisms and this matrix provides structural stability to the biofilm. The biofilm structure provides protection to the cells against host-defense mechanisms, phagocytosis, biocides, hydrodynamic shear forces and antibiotic treatment. Biofilm is considered to be responsible for 65% of all bacterial infections (Potera, 1999).

Biofilm formation is developed in three main stages (Figure 2.8):

(1) Attachment, the cells arrive to the surface and adhere to this surface;
(2) Growth and maturation, they begin to produce the exopolysaccharide that constitutes the matrix and mature from microcolonies to multilayered cell clusters;
(3) Detachment, the cells take on a planktonic state and can thereby form biofilm in other settings.

It has been proposed that detachment mechanisms can be divided into two categories: active and passive. Active detachment refers to mechanisms initiated by the bacteria themselves, such as enzymatic degradation of the biofilm matrix, quorum sensing, etc. On the other hand, passive detachment refers to that mediated by external forces such as fluid shear, abrasion and human intervention. It has also been proposed that the detachment process may be caused by bacteriophage activity within the biofilm (Soto, 2013).

![Figure 2.8 Steps in biofilm formation](Soto, 2013)

### 2.14 Mechanisms of Antimicrobial Resistance in Bacteria inside the Biofilm Structures

One of the main properties of bacteria in biofilms is their capacity to be more resistant to antimicrobial agents than planktonic cells. This feature makes it difficult to eradicate infections caused by biofilm forming bacteria, constituting a serious clinical problem (Jucker et al 1991). Biofilm structures show maximum resistance to antibiotics in the mature stage.

**Several mechanisms are reportedly responsible for the antimicrobial resistance in biofilm structures:**

1. Poor diffusion of antibiotics through the biofilm polysaccharide matrix, although some antibiotics are able to penetrate the matrix; (Anderl et al 2000)
2. Physiological changes due to slow growth rate and starvation responses (Oxygen, nutrient deprivation or environmental stress); (Walters et al 2003)
(3) Phenotypic change of the cells forming the biofilm;
(4) Quorum-sensing, although their exact role is not clear; (Brooun et al 2000)
(5) The expression of efflux pumps; (Gilbert et al 2002)
(6) Persister cells: small fractions of persistent bacteria that resist killing when exposed to antimicrobials. The persistent cells are not mutants (Kim, 2006).

2.15 Why Antimicrobials fail Resistance & Tolerance

The minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) are standard values in antibiotic susceptibility testing and serve as important references in the treatment of acute infections. Specifically, they assess the effect of antibiotics against planktonic organisms in exponential growth. In biofilms, the MBC may be three to four logs higher compared with exponential planktonic cells (Ceri et al 1999; Anderl et al 2000). Bacterial growth inhibition within a biofilm is poorly evaluated. Most studies have relied upon conventional MIC testing based on optical density, which is more reflective of the prevention of growth of planktonic bacteria shed from the biofilm, than of cell growth within the biofilm. Thus, it is not necessarily surprising that similar MIC values have been reported for biofilms and planktonic cultures (Ceri et al 1999). Biofilms are highly tolerant to antibiotics in terms of killing but have not been shown to be much more resistant to growth inhibition than exponential planktonic cells. The standardization of a minimum biofilm eradicating concentration (mBEC) has been postulated in the attempt to correlate in vitro measurements with therapeutic outcomes in biofilm treatment (Anwar et al 1992).

Interestingly, bacteria rapidly regain their antibiotic susceptibility after they have been mechanically dispersed from the biofilm architecture and transferred in fresh medium (Anderl et al 2003; Williams et al 1997). This underscores that antibiotic tolerance within biofilms is not acquired via mutations or mobile genetic elements but represents a functional characteristic of biofilm formation. Disruption of the biofilm may allow cells previously starving in deep layers new access to nutrients, which rapidly (i.e., within a few hours) brings them into exponential growth phase and subsequently renders them susceptible to antibiotics. Alternatively, disruption of the biofilm may result in the dilution of cell signals, which mediate metabolic inactivity and antibiotic resistance within a biofilm.
2.16 Antimicrobial susceptibility & growth phase

Virtually all antimicrobials are more effective at killing rapidly growing than stationary cells (Eng et al 1991). Some antibiotics, such as penicillin and ampicillin, have an absolute requirement for cell growth in order to kill (Tuomanen et al 1986). Eng and colleagues were able to demonstrate, by controlling the growth rate of bacteria through nutrient limitation, that only fluoroquinolones produced bactericidal effects against stationary-phase Gram-negative organisms (Eng et al 1991). No class of antimicrobial agents was bactericidal in growth-limited S. aureus. Antibiotic tolerance was found to be similar in biofilms and stationary-phase planktonic cultures of P. aeruginosa (Spoering & Lewis, 2001). The comparable concentrations of catalase and a stationary phase sigma factor in both cultures suggest that at least some bacteria in biofilms exhibit stationary phase characteristics (Anderl et al 2003; Xu et al 2001). The antimicrobial tolerance of biofilms may thus be due to analogous metabolic and reproductive inactivity. The bacteria’s ‘dormant state’ could be triggered by substrate depletion and accumulation of inhibitory waste products.

2.17 Antimicrobials fail to penetrate biofilms

The diffusion of antibiotics through biofilms has been assessed by concentration measurements and visualization of bactericidal effects in the depths of in vitro biofilms (Anderl et al 2000; Anderl et al 2003). Most studies have documented unimpaired antimicrobial penetration (Anderl et al 2000; Zheng & Stewart, 2002). Although antibiotic transport into biofilms is not the limiting step in general, three exceptions have been noted. In a β-lactamase-positive K. pneumoniae biofilm, β-lactam antibiotics were deactivated in the surface layers more rapidly than they diffused (Anderl et al 2003). Second, the biofilm penetration of the positively charged aminoglycosides is retarded by binding to the negatively charged matrix polymers (Walters et al 2003). This retardation may allow more time for bacteria to implement adaptive stress responses. Aminoglycoside penetration into a P. aeruginosa biofilm was significantly hindered by binding to the extracellular alginate but markedly improved after the addition of alginate lyase (Gordon et al 1988). Third, extracellular slime derived from coagulase-negative staphylococci reduced the activity of glycopeptides even in planktonic bacterial cultures (Konig et al 2001; Souli & Giamarellou, 1998).
2.18 Quorum sensing & biofilms

Many bacteria communicate via the production and sensing of autoinducer ‘Pheromones’ in order to control the expression of specific genes in response to population density. This so-called quorum sensing is widely used to co-ordinate gene expression within a species (Diggle, Stephen et al 2002). The bioluminescent marine bacterium *Vibrio harveyi* regulates its light production in response to cell density. Its transmitter, the autoinducer AI-2, has been found to allow interspecies communication between Gram-positive and -negative bacteria (Bassler et al 1993). Given the tremendous metabolic and structural changes associated with the switch from planktonic growth to growth within a mature biofilm community, it seems reasonable that cell–cell signaling regulates biofilm formation. In 1998, quorum sensing was found to modulate the transformation of *P. aeruginosa* from planktonic to a biofilm mode of growth (Davies et al 1998).

2.19 The Effect of Efflux Pump Inhibitors on Biofilm

Efflux-pump inhibitors (EPIs) are substances that inhibit the flux of substances mediated by efflux pumps. These efflux pumps are considered as important drug targets for the development of combination strategies using antibiotic efflux inhibitors (Mahamoud et al 2007). EPIs are generally simple, robust and cheap chemicals which are well tolerated by humans (Marquez, 2005). EPI compounds must show the following criteria:

1. They must enhance the activity of the pump substrates;
2. They should not show activity in efflux pump mutants;
3. They must increase the accumulation and decrease the extrusion of efflux pump substrates;
4. They must not affect the proton gradient across the cytoplasmic membrane.

**2.20 Inhibition of this efflux activity may be performed in different ways:**

1. Alternating the regulatory steps in the expression of efflux pumps,
2. Inhibiting the functional assembly of the multi-component pump,
3. Blocking the outer membrane channel (TolC, OprM) with a plug,
4. Collapsing the energy of efflux,
5. Creating competitive or non-competitive inhibition with a nonantibiotic molecule to the affinity sites of the efflux pump,
6. Changing the chemical design of previous antibiotics to reduce the affinity for efflux recognition and binding sites. (Pages & Amaral, 2009)
Some EPIs can also inhibit bacterial biofilm formation. Thus, Thioridazine, Phe-Arg β-naphthylamide (PAβN) or the Arylpirazine NMP are some of the compounds categorized as efflux pump inhibitors. It has been observed that the addition of these compounds significantly reduced formation of biofilm in several bacteria, such as *E. coli*, *Klebsiella pneumonia*, *S. aureus* and *P. putida* (Kvist et al 2008). PAβN was the first EPI identified and, in combination with fluoroquinolones, showed inhibitory capacity against AcrAB-TolC, MexAB-OprM, MexCD-OprJ and MexEF-OprN pumps (Lomovskaya O, 2001). PAβN and CCCP (a proton motive force uncouple) had great ability to repress biofilm formation by the inhibition of efflux pumps (Baugh et al 2012).

EPIs from natural sources have also shown antibiofilm activity. Thus, the natural EPI caffeoylquinic acid (CQA) from *Artemisa absinthium* reduces biofilm viability in combination with subinhibitory concentrations of ethidium bromide and moxifloxacin in *S. aureus* and *Enterococcus faecalis*. This EPI acts by enhancing the killing effect of these compounds (Fiamegos et al 2011). Therefore, due to their characteristics, EPIs could be used as enhancers of the antibiotics used in the treatment of biofilm (Kvist et al 2008). In summary, there is currently insufficient knowledge as to the role of efflux pumps in biofilm resistance and, thus, further studies are needed to elucidate the role of these systems in bacterial biofilms. However, the existing studies show that efflux pumps could be an attractive target for antibiofilm drug development (Soto, 2013).